INOSITOL 1,4,5-TRISPHOSPHATE AND INTRACELLULAR Ca^{2+} STORE SITES IN HUMAN PERIPHERAL LYMPHOCYTES

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ABSTRACT

Ca^{2+} storage by vesicles in human lymphocytes was studied using saponin-permeabilized cells. Saponin-treated lymphocytes could accumulate Ca^{2+} in the presence of ATP. In the presence of NaN_3 (10 mM), the maximal storage of Ca^{2+} (0.87 nmol/ 4×10^6 cells) was obtained at free Ca^{2+} concentration of 10^{-5} M, and the apparent affinity constant for Ca^{2+} was approximately $1.4 \times 10^6 \text{ M}^{-1}$. Stored Ca^{2+} was rapidly released by A23187. Ca^{2+} accumulation was markedly enhanced by oxalate, and precipitates of Ca-oxalate were electron-microscopically observed inside the endoplasmic reticulum-like structures. In the absence of NaN_3 , the maximal amount of intracellular Ca^{2+} was about 30 nmol/ 4×10^6 cells, while the affinity for Ca^{2+} was much lower. Application of inositol 1,4,5-trisphosphate to saponin-permeabilized cells which had accumulated Ca^{2+} by ATP in the presence of NaN_3 rapidly released 35% of stored Ca^{2+} within 1 min. However, when the concentration of free Ca^{2+} was higher than 1.5×10^{-6} M, Ca^{2+} release by this agent was inhibited. The results suggest that inositol 1,4,5-trisphosphate may function as a mediator in lymphocytes, linking activation of surface membrane receptors with mobilization of internal Ca^{2+} , which plays an important role in lymphocyte responses involved in proliferation.

In lymphocytes, mitogenic lectins such as concanavalin A (ConA) or phytohemagglutinin (PHA) can induce a rapid increase in the cytoplasmic free Ca^{2+} concentration, which may act as one of the primary signals for the transition from the resting state to the proliferating state (5, 14, 16). The Ca^{2+} contributing to the activation of lymphocytes is derived from both extracellular and intracellular origins. For example, when the mouse thymocytes were stimulated with ConA, the cytoplasmic free Ca^{2+} concentration increased from 105 nM to 250 nM or to 160 nM in the presence or absence of extracellular Ca^{2+} ,

respectively (9). Thus, intracellularly stored Ca^{2+} may partly contribute to the increase in the cytoplasmic free Ca^{2+} in lymphocytes as induced by ConA. However, there is no documentation as to the intracellular Ca^{2+} pool in lymphocytes.

Recently, it has been reported that inositol 1,4,5-trisphosphate (InsP_3), a metabolite of membrane phosphatidyl-inositol 4,5-bisphosphate (PI-P_2) induced a release of Ca^{2+} from non-mitochondrial Ca^{2+} store sites in various types of cells (2, 3, 13, 22, 23). Thus, InsP_3 is considered to act as a mediator between the surface receptor and the intracellular Ca^{2+} store sites.

In the present study, we present evidence for the existence of ATP-dependent Ca^{2+} accumulating system in human peripheral lymphocytes. We further examined the effect of InsP_3 on the release of Ca^{2+} from the ATP-dependent Ca^{2+} pool.

Abbreviations: EGTA, ethyleneglycol bis-(2-amino-ethylether)-*N,N'*-tetraacetate; PI-P_2 , phosphatidylinositol 4,5-bisphosphate; InsP_3 , inositol 1,4,5-trisphosphate

MATERIALS AND METHODS

Preparation of Saponin-treated Lymphocytes

Human peripheral lymphocytes were obtained from mononuclear cell-rich plateletpheresis residues of healthy donors (21). The mononuclear cell-rich residues were diluted with 0.9% NaCl and were sedimented at 200 g for 5 min. The platelet-rich supernatant was discarded. The sedimented cells were placed on a density gradient of Ficoll-Hypaque. After centrifugation (450 g, 15 min), cells at the interface were washed with a medium of RPMI 1640 supplemented with 20 mM Hepes (pH 7.40) and 5% fetal calf serum, and then passed through a column of nylon fiber in order to separate from contaminating adherent cells. Differential cell determination based on morphologic criteria indicated that more than 95% of cells were lymphocytes. Saponin treatment was carried out by essentially the same method as described (12, 23). In brief, 1×10^8 cells were incubated in a solution (5 ml) containing 130 mM KCl, 20 mM Tris-maleate (pH 6.80), 5 mM $MgCl_2$, 5 mM ethyleneglycol bis-(2-aminoethylether)-*N,N'*-tetraacetate (EGTA), 2 mM diisopropylfluorophosphate, 0.2% bovine serum albumin (BSA) and 100 μg /ml saponin for 5 min at 37°C. After centrifugation (200 g, 5 min), cells were suspended in a solution of 100 mM KCl, 20 mM Tris-maleate (pH 6.80) and 0.2% BSA.

Assay of Ca^{2+} Uptake and Release

Ca^{2+} uptake and release were assayed by a filtration method using ^{45}Ca . The reaction mixture contained 100 mM KCl, 20 mM Tris-maleate (pH 6.80), 3 mM $MgCl_2$, 10 mM NaN_3 , 0.12 mM $^{45}CaCl_2$ (5,000–6,000 cpm/nmol), specified concentration of EGTA to obtain the desired free Ca^{2+} concentration, and 4×10^6 cells/ml. The reaction was started by the addition of 3 mM ATP and the reaction mixture was incubated at 37°C for various times. At the desired time, an aliquot of the mixture was passed through a glass-fiber filter (Whatman GF/C, pore size 1.2 μm). The filter was washed twice with the above mixture without ^{45}Ca , dried and then counted for radioactivity in 10 ml of a scintillation cocktail (4 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis(5-phenyl 2-oxazolyl)benzene in 1 l of toluene). Thus the amount of Ca^{2+} uptake was determined. In other experiments, $InsP_3$ in 1/100 vol of the reaction mixture was added. At indicated times, Ca^{2+} remaining in saponin-treated cells was determined as described above.

Apparent binding constant of EGTA for Ca^{2+} was assumed to be $1 \times 10^6 M^{-1}$ at pH 6.80 (6).

Ca^{2+} Uptake by Saponin-treated Lymphocytes as Seen by Electron Microscopy

Ca^{2+} uptake was examined by electron microscopy as described (13). Saponin-treated lymphocytes (4×10^7 cells) were incubated for 30 min at 37°C in 10 ml of a medium containing 100 mM KCl, 20 mM Tris-maleate (pH 6.80), 3 mM $MgCl_2$, 0.12 mM $CaCl_2$, 10 mM potassium oxalate, 3 mM ATP, 10 mM creatine phosphate plus 2 units/ml of creatine phosphokinase (Sigma, type 1) and 10 mM NaN_3 . After centrifugation at 250 g for 10 min, the cell pellet was fixed with 2.4% (w/v) glutaraldehyde, and treated as described previously (17).

Materials

$^{45}CaCl_2$ was the product of New England Nuclear. Saponin was obtained from I.C.N. Nutritional Biochemicals. Other reagents were commercial preparations of reagent grades. $InsP_3$ was prepared from erythrocyte ghosts by the method of Downes *et al.* (4).

RESULTS

Time Course of Ca^{2+} Uptake by Saponin-treated Lymphocytes and Effects of Oxalate and A23187 on the Ca^{2+} Uptake

Fig. 1 shows the time course of Ca^{2+} uptake by saponin-treated lymphocytes in the presence of NaN_3 , an inhibitor of mitochondrial Ca^{2+} uptake (10, 12, 13). By the addition of ATP, saponin-treated lymphocytes could accumulate Ca^{2+} , and the Ca^{2+} accumulation was reached a steady-state within 5 min. The addition of A23187 (1 μM), a Ca^{2+} -ionophore, rapidly released all the accumulated Ca^{2+} . The inclusion of oxalate, a Ca^{2+} -precipitating anion, in the reaction mixture markedly enhanced the accumulation of Ca^{2+} . For example 15 min after the addition of ATP, the amount of accumulated Ca^{2+} was increased from 0.9 to 3.4 nmol/ 4×10^6 cells by the inclusion of 10 mM oxalate. These results indicate the existence of the ATP-dependent and non-mitochondrial Ca^{2+} store site in human lymphocytes, and also that the Ca^{2+} store is a vesicular structure.

Site of the Ca^{2+} Uptake by Saponin-treated Lymphocytes in the Presence of NaN_3

Oxalate stimulated the Ca^{2+} uptake by saponin-

Fig. 1 Time course of ATP-dependent Ca^{2+} uptake by saponin-treated lymphocytes. Ca^{2+} uptake was determined as described in Materials and Methods. Δ , ATP free; $\circ$, 3 mM ATP; $\bullet$, 3 mM ATP plus 10 mM potassium oxalate; and $\blacksquare$, 1 μM A23187. The vertical bars represent the SEM in four independent experiments.

treated lymphocytes as described above. This stimulation by oxalate may be due to the formation of calcium-oxalate precipitates in vesicles (17). Therefore, we examined electron-microscopically the precipitates of calcium-oxalate to identify the site of the Ca^{2+} uptake in the presence of NaN_3 . As shown in Fig. 2, the precipitates of calcium-oxalate were observed in the endoplasmic reticulum-like structures. In the absence of NaN_3 , the precipitates were observed in both mitochondria and endoplasmic reticulum-like structures (data not shown). Although the precipitates were observed as totally electron-dense deposits in the preparation stained with uranyl acetate and lead (Fig. 2A), the precipitates were recognized as bubble-like deposits in the negatively stained sample (Fig. 2B). These observations were much the same as those obtained with guinea-pig macrophages and brain microsomes (13, 15).

Ca^{2+} Uptake by Saponin-treated Lymphocytes at Various Concentrations of Free Ca^{2+} in the Presence of NaN_3

We next examined the Ca^{2+} uptake by the non-mitochondrial, endoplasmic reticulum-like vesicles at various concentrations of free Ca^{2+}

(Fig. 3). The amount of Ca^{2+} uptake by endoplasmic reticulum-like vesicles increased sigmoidally at free Ca^{2+} concentrations ranging from 3×10^{-8} to 5×10^{-5} M, and the maximal capacity of Ca^{2+} uptake was $0.87 \text{ nmol}/4 \times 10^6$ cells. The apparent binding constant for the Ca^{2+} uptake, which is the reciprocal of the free Ca^{2+} concentration for the half-maximal Ca^{2+} uptake, was about $1.4 \times 10^6 \text{ M}^{-1}$, such being comparable with the values in guinea-pig macrophages (12), dog tracheal smooth muscle (8) or porcine coronary arterial smooth muscle (24). The amount of ATP-independent Ca^{2+} binding was also measured, but the value was much low.

Ca^{2+} Uptake by Saponin-treated Lymphocytes at Various Concentrations of Free Ca^{2+} in the Absence of NaN_3

Fig. 4 shows the Ca^{2+} uptake by saponin-treated lymphocytes at various concentrations of free Ca^{2+} in the absence of NaN_3 . The maximal Ca^{2+} uptake was about $30 \text{ nmol}/4 \times 10^6$ cells seen at free Ca^{2+} concentration of 10^{-4} M, and this value was approximately 33 times larger than that obtained in the presence of NaN_3 . Since the precipitates of calcium-oxalate were observed in both mitochondria and endoplasmic reticulum-

Fig. 2

Fig. 3 Effects of free Ca^{2+} concentrations on the Ca^{2+} uptake by saponin-treated lymphocytes. ○, ATP-free; ●, 3 mM ATP. Vertical bars represent the SEM in ten independent experiments.

Fig. 4 Ca^{2+} uptake by saponin-treated lymphocytes at various concentrations of free Ca^{2+} . ○, 10 mM NaN_3 ; ●, NaN_3 -free. Vertical bars represent the SEM in four independent experiments.

Fig. 2 Electron micrographs of saponin-treated lymphocytes that have accumulated Ca^{2+} in the presence of 10 mM oxalate. The precipitates of calcium-oxalate are indicated by arrows. A: Uranyl acetate and lead staining. B: Negative staining. A, $\times 17,000$. B, $\times 40,000$. Inset in B, $\times 45,000$

Fig. 5 Time course of Ca^{2+} release induced by InsP_3 from intracellular store sites in saponin-treated lymphocytes. Ca^{2+} release was determined as described in Materials and Methods. Free Ca^{2+} concentration in the reaction mixture was 3.7×10^{-7} M. ○, control; ●, 5 μM InsP_3 ; ■, 1 μM A23187; and △, ATP-free. A typical example in four independent experiments is shown.

like vesicles in the absence of NaN_3 , Ca^{2+} must have been taken up by these two kinds of organelles. At free Ca^{2+} concentration lower than 1.5×10^{-6} M, the amount of Ca^{2+} uptake in the absence of NaN_3 was much the same as that obtained in the presence of NaN_3 , indicating that the threshold concentration of free Ca^{2+} for mitochondrial Ca^{2+} uptake is higher than 1.5×10^{-6} M. Thus, although the mitochondrial Ca^{2+} uptake has a high capacity, its affinity for free Ca^{2+} is much lower, when compared with the non-mitochondrial Ca^{2+} uptake.

InsP₃ Releases Ca^{2+} from Saponin-treated Lymphocytes

Fig. 5 shows the time course of Ca^{2+} release induced by InsP_3 or A23187 from the non-mitochondrial Ca^{2+} store site of saponin-treated lymphocytes. When saponin-treated lymphocytes were incubated in a solution containing 3.7×10^{-7} M free Ca^{2+} (with 5 mM MgCl_2 plus 3 mM ATP) for 10 min, the amount of Ca^{2+}

stored in cells was about $0.32 \text{ nmol}/4 \times 10^6$ cells. InsP_3 (5 μM) or A23187 (1 μM) was added 11 min after the addition of ATP, to release the stored Ca^{2+} . InsP_3 (5 μM) released Ca^{2+} within 1 min, and the amount of released Ca^{2+} was about 35% of the stored Ca^{2+} . In contrast, A23187 (1 μM) released all the stored Ca^{2+} within 1 min. InsP_3 at 5 μM showed the maximal effect (data not shown). The InsP_3 -induced release of Ca^{2+} was modified by the concentration of free Ca^{2+} . As shown in Fig. 6, the release of Ca^{2+} induced by InsP_3 was inhibited when Ca^{2+} was loaded in cells at the concentrations of free Ca^{2+} over 1.5×10^{-6} M, despite that much more Ca^{2+} was stored in cells.

DISCUSSION

In this study, we present evidence for the existence of ATP-dependent Ca^{2+} accumulating system in human peripheral lymphocytes. The Ca^{2+} -storing site in the presence of NaN_3 seems to be endoplasmic reticulum-like vesicles,

Fig. 6 Effects of free Ca^{2+} concentrations on the Ca^{2+} release induced by $5 \mu\text{M}$ InsP_3 . Ca^{2+} release was measured 1 min after the addition of $5 \mu\text{M}$ InsP_3 essentially as described in Materials and Methods. A typical example in four independent experiments is shown.

because the Ca^{2+} accumulation was markedly enhanced by oxalate, and the precipitates of Ca -oxalate were observed inside the endoplasmic reticulum-like vesicles by electron microscopy. Ca^{2+} accumulation in the presence of NaN_3 was seen at free Ca^{2+} concentrations ranging from 10^{-8} M to 10^{-5} M, and apparent affinity for free Ca^{2+} was high ($1.4 \times 10^6 \text{ M}^{-1}$). These values were close to those obtained with the skeletal and cardiac sarcoplasmic reticulum (11, 20). On the other hand, Ca^{2+} accumulation in the absence of NaN_3 seems to be due mainly to mitochondria; affinity for free Ca^{2+} of mitochondria was much lower than that of the non-mitochondrial system. Since the amount of Ca^{2+} accumulation at free Ca^{2+} concentration lower than 1.5×10^{-6} M in the absence of NaN_3 was much the same as that obtained in the presence of NaN_3 , the threshold Ca^{2+} concentration for mitochondrial Ca^{2+} accumulation may be higher than 1.5×10^{-6} M. The cytoplasmic free Ca^{2+} concentration in unstimulated lymphocytes as assessed with quin II-loaded cells is around 1×10^{-7} M, and this increases to 1.6 – 2.5×10^{-7} M by stimulation with lectins (9). Therefore, the non-mitochondrial, endoplasmic reticulum-like structure may mainly function as a physiological intracellular Ca^{2+} pool.

Mitogenic lectins can rapidly increase the

cytoplasmic free Ca^{2+} concentration in lymphocytes, and the Ca^{2+} increase is moderately reduced when extracellular Ca^{2+} is deprived by EGTA (9). Thus, the intracellular Ca^{2+} pool in addition to the extracellular pool may also contribute to the change in the free Ca^{2+} concentration in lymphocytes. Recently, it has been reported that the turnover of inositol phospholipids is involved in the activation of receptors by agonist in many tissues including lymphocytes (7, 18, 19). We observed here that micromolar concentration of InsP_3 induced a rapid release of Ca^{2+} from the non-mitochondrial, endoplasmic reticulum-like vesicles in human peripheral lymphocytes. Therefore, InsP_3 may be a mediator linking between mitogen-stimulation on the surface membrane and non-mitochondrial internal Ca^{2+} pool. Further studies are now required to examine whether the time course and amount of InsP_3 production are correlated with those of the increase in the cytoplasmic free Ca^{2+} concentration in human peripheral lymphocytes stimulated with mitogenic lectins.

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