Mitogenic stimulation transiently increases the exchangeable mitochondrial calcium pool in rat thymocytes

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Exchangeable calcium pools were measured in rat thymocytes by ^{45}Ca labelling and selective depletion of intracellular pools with oligomycin in the presence or absence of rotenone. The mitochondrial pool increased by 150% after 3 min of treatment with the mitogen concanavalin A, and decreased to zero 10 min after mitogen addition. No significant change in the ATP-dependent pool could be detected.

INTRODUCTION

A number of growth factors and mitogens have been shown to activate a signal-transduction pathway which includes the production of inositol polyphosphates and the consequent mobilization of calcium [1]. The mitogen concanavalin A (ConA) produces a rapid increase in cytoplasmic free Ca^{2+} in mouse and rat thymocytes [2,3] and an increase in inositol 1,4,5-trisphosphate (IP_3) in mouse thymocytes [2]. In permeabilized rat thymocytes, IP_3 releases Ca^{2+} from an intracellular ATP-dependent pool [3].

This pathway is apparently very similar to the signal-transduction mechanism used by calcium-mobilizing hormones in liver [4], in which release of Ca^{2+} from an intracellular ATP-dependent pool (assumed to be the endoplasmic reticulum) and changes in Ca^{2+} transport across the plasma membrane have been implicated. The role of the mitochondrial calcium pool has been disputed, some workers proposing it as a source of mobilized calcium [5] and others as a sink for buffering cytoplasmic free Ca^{2+} [6]. It has subsequently been proposed that an increase in mitochondrial Ca^{2+} serves to activate mitochondrial dehydrogenases [7,8], and Assimacopoulos-Jeannet *et al.* [9] have demonstrated an increase in total mitochondrial calcium and a parallel activation of dehydrogenases after hormonal stimulation of rat liver.

The role of the mitochondrial calcium pool in mitogenic stimulation has not been as thoroughly studied, although analogies to hormonal stimulation of liver have been suggested. Ardawi & Newsholme [10] have proposed that the increase in cytoplasmic Ca^{2+} after mitogenic stimulation of lymphocytes may also increase mitochondrial Ca^{2+} and activate 2-oxoglutarate dehydrogenase. An activation of pyruvate dehydrogenase in the absence of changes in other known effectors of the enzyme has been observed in pig lymphocytes stimulated by ConA in our laboratory [11], and it was suggested that an increase in mitochondrial Ca^{2+} could account for this activation. In the experiments described below we have estimated the size of the exchangeable mitochondrial calcium pool in rat thymocytes and have demonstrated an increase in the size of this pool after ConA stimulation.

EXPERIMENTAL

Preparation of thymocytes

Thymocytes were prepared from 4–6-week-old female Wistar rats. The thymus was disaggregated by pressing through nylon mesh, and the cells were washed once by centrifugation at 1000 *g* for 5 min and resuspended at 4×10^7 – 5.5×10^7 cells/ml at 37 °C. Incubation medium was RPMI 1640 with 2 mM-glutamine (obtained in powdered form from Flow Laboratories), buffered at pH 7.4 with 10 mM-Hepes and 24 mM- NaHCO_3 , supplemented with 20 μg of gentamicin/ml, and filtered through a 0.2 μm -pore-size filter to remove undissolved particles. Cells were incubated in plastic flasks at 37 °C with gentle shaking under an atmosphere of CO_2 /air (1:19). Viability as determined by Trypan Blue exclusion was > 95% in freshly isolated cells.

^{45}Ca labelling of thymocytes

Thymocytes in RPMI were labelled with 1.5 μCi of ^{45}Ca /ml (3.5 mCi/mmol final specific radioactivity) for 60 min at 37 °C, and $^3\text{H}_2\text{O}$ was added to 7.5 μCi /ml before addition of ConA or inhibitors as indicated below. After appropriate incubations, portions of cell suspensions (0.25 ml) were rapidly pipetted into 1.5 ml microfuge tubes at 4 °C containing 50 μl of 20% (v/v) Triton X-100/0.25 M-sucrose overlaid with 250 μl of oil [42% (v/v) dinonyl phthalate/48% silicone fluid D.C. 550, both obtained from BDH] and 250 μl of EGTA wash medium (RPMI buffered with 20 mM-Hepes, pH 7.4, and containing 4 mM-EGTA, 20 μg of gentamicin/ml, 10 μM -Ruthenium Red purified as described in ref. [12] and 7.5 μCi of $^3\text{H}_2\text{O}$ /ml). Centrifuge tubes were pre-loaded into an Eppendorf microfuge with swinging-bucket rotor, samples were rapidly pipetted into the tubes, and centrifugation was started within 10 s to minimize the time of exposure of cells to the EGTA wash. After centrifugation for 60 s at 10000 *g*, 250 μl of the supernatant was removed and the centrifuge tube was frozen in a solid- CO_2 /ethanol bath. The tube was cut through the oil layer to remove the entire Triton/sucrose pellet, and the cell pellet was resuspended by vortex-mixing with water in a scintillation vial. Radioactivity in supernatants and pellets was determined by dual-channel liquid-scintillation counting for ^{45}Ca and ^3H by using

quench and cross-over corrections. Counting error was < 8%.

Calculation of intracellular calcium

Total pellet ^{45}Ca and ^3H radioactivity was corrected by subtracting extracellular radioactivity, estimated as described below. The ratio of $^{45}\text{Ca}/^3\text{H}$ in pellets was corrected to intracellular [calcium] in $\mu\text{mol/l}$ of cell water by making the following assumptions: (1) the corrected pellet radioactivity represents only the intracellular space; (2) the intracellular concentration of $^3\text{H}_2\text{O}$ is the same as that in the medium; (3) ^{45}Ca approaches isotopic equilibrium with the exchangeable calcium pools after 60 min of labelling. Assumption (3) was based on ^{45}Ca -uptake studies, which showed a rapid initial uptake rate which declined after 60 min. ^{45}Ca in the pellets continued to increase slowly for more than 24 h, but this may be due to the declining viability of the cells and the uptake of excess calcium by dying cells. After 60 min of labelling, intracellular calcium in untreated cells was determined to be approx. $160 \mu\text{mol/l}$ of cell volume.

Extracellular volume was estimated in a series of control incubations at 4°C . Thymocytes were labelled with $7.5 \mu\text{Ci}$ of $^3\text{H}_2\text{O}/\text{ml}$ and ^{45}Ca was added to $1.5 \mu\text{Ci}/\text{ml}$ for 10 s, followed by centrifugation through EGTA wash medium and determination of radioactivity as described above. The pellet ^{45}Ca was used to estimate extracellular volume, on the assumption that ^{45}Ca uptake at 4°C would be negligible after 10 s. This method also corrects for extracellular bound ^{45}Ca not removed by the EGTA wash. From a number of samples of different cell density, an empirical relationship was determined between total pellet volume (^3H in the pellet as a percentage of supernatant ^3H) and extracellular volume (^{45}Ca in the pellet as a percentage of supernatant ^{45}Ca), and this function was subsequently used to calculate extracellular volume from the total pellet volume.

Mitogen and inhibitor incubations

ConA was dissolved in RPMI medium and added to cell suspensions (4×10^7 – 5.5×10^7 cells/ml) at $2.5 \mu\text{g}/\text{ml}$ final concn. (4.5 – $6.3 \mu\text{g}/10^8$ cells). The optimal concentration of ConA for mitogenic stimulation, assayed by [^3H]thymidine uptake after 48 h as described by

Moore *et al.* [13], was found to be 0.3 – $0.45 \mu\text{g}/\text{ml}$ for cells at 6×10^6 cells/ml, or 5 – $7.5 \mu\text{g}/10^8$ cells.

Mitochondrial inhibitors oligomycin and rotenone were added to cell suspensions as DMSO stock solutions at final concentrations of $10 \mu\text{g}$ of oligomycin/ml, $5 \mu\text{M}$ -rotenone and 0.83% DMSO. In control incubations, DMSO was added to the same final concentration. These concentrations of oligomycin and rotenone produced maximal inhibition of respiration as determined in an oxygen electrode.

ATP assays

Total ATP was determined in cell extracts prepared by precipitating $250 \mu\text{l}$ of cell suspensions with $500 \mu\text{l}$ of 3% (w/v) HClO_4 and neutralizing the extract with $300 \mu\text{l}$ of 1 M-KOH in 1 M-Hepes . Cell extracts were assayed by using firefly lantern extract (Sigma) in a DuPont Luminescence Biometer according to the manufacturer's directions, and cellular ATP was calculated from a standard curve obtained with ATP treated as for the cell extract.

RESULTS AND DISCUSSION

The size of the mitochondrial calcium pool in rat thymocytes has been estimated by the use of inhibitors which are expected to act selectively either on the mitochondrial calcium pool or on the ATP-dependent calcium pool. Oligomycin, by inhibiting the mitochondrial ATPase, should deplete cellular ATP without releasing the mitochondrial pool, which is dependent on the mitochondrial membrane potential, whereas rotenone should decrease this potential and release the mitochondrial pool. In synaptosomes it has been shown that oligomycin + rotenone collapse the potential, whereas oligomycin alone slightly increases it [14]. With rotenone alone, the membrane potential may be maintained by the hydrolysis of ATP by the mitochondrial ATPase [14]. We have therefore determined the effects of rotenone in the presence of oligomycin.

The effects of these inhibitors on total exchangeable calcium in thymocytes are described by Fig. 1. Inhibitors (oligomycin with or without rotenone) were added at zero time, and samples were removed at 5 min intervals

Fig. 1. Time course of inhibitor effects on thymocyte calcium

Thymocytes were labelled with ^{45}Ca and treated with DMSO (Δ), oligomycin ($\square$) or oligomycin + rotenone ($\blacksquare$) as described in the Experimental section. Inhibitors were added at zero time, and samples were removed at 5 min intervals for determination of intracellular calcium as described. Cells in panel (b) were pre-treated with ConA for 3 min before addition of inhibitors. Results shown are means $\pm$ S.E.M. for a total of three to five determinations from three to five separate thymocyte preparations.

Fig. 2. Thymocyte calcium after 5 min inhibitor treatments

Thymocytes were labelled with ^{45}Ca and treated with DMSO, oligomycin or oligomycin + rotenone as indicated. Samples were removed after 5 min for determination of intracellular calcium as described in the Experimental section. Cells were pre-treated with or without ConA for 3 min before addition of inhibitors as indicated: $\square$, DMSO; ▨ , oligomycin; $\blacksquare$, oligomycin + rotenone. The rotenone-releasable calcium pool (b) was calculated as the difference between oligomycin and oligomycin + rotenone treatments. Results shown are means $\pm$ S.E.M. for a total of 12–22 determinations from six to ten separate thymocyte preparations.

for determination of intracellular calcium. In control incubations (Fig. 1a), oligomycin released approx. 30% of the total calcium at 5 min and 50% by 20 min; rotenone released an insignificantly small amount of additional calcium. In Fig. 1(b) cells were pre-treated with ConA for 3 min before addition of inhibitors. After ConA treatment, rotenone released a larger amount of calcium at 5 min, and no additional calcium was released by longer exposure to rotenone. The rotenone-releasable pool, defined as the difference between treatments with oligomycin and with oligomycin + rotenone, did not change significantly between 5 and 20 min, and therefore 5 min inhibitor treatments were used in subsequent experiments to estimate the size of this pool.

Fig. 3. Thymocyte ATP after 5 min inhibitor treatments

Thymocytes were treated with DMSO ($\square$), oligomycin (▨) or oligomycin + rotenone ($\blacksquare$) as indicated, and samples were removed after 5 min for assay of total ATP as described in the Experimental section. Cells were pre-treated with or without ConA for 3 min before addition of inhibitors as indicated. Results shown are means $\pm$ S.E.M. for a total of three to nine determinations from three to five separate thymocyte preparations. ATP in DMSO controls was approx. 60 nmol/ 10^8 cells, or 7.5 nmol/l of cell water.

A larger number of 5 min inhibitor incubations are summarized in Fig. 2. Pretreatment for 3 min with ConA was found to increase calcium in the DMSO controls (significant at $P < 0.025$ by Student's t test). Assuming the cells were close to isotopic equilibrium, this represents a net influx of calcium and is similar to increases seen in rat splenocytes [15,16] and human T-lymphocytes [17]. As shown in Fig. 2(b), ConA increased the rotenone-releasable calcium pool by $> 150\%$ (significant at $P < 0.01$ by Student's t test). If rotenone was replaced by myxothiazol (1 μM final concn.), an increase in the myxothiazol-releasable pool was also seen after ConA treatment (results not shown), indicating that the effects of both inhibitors are due to their common action on mitochondrial electron transport.

The possibility that rotenone released additional calcium by further depletion of ATP was examined by assaying ATP concentrations (Fig. 3) under the same conditions used for calcium assays. Total ATP was determined after incubations with ConA and/or inhibitors as in Fig. 2, and no significant differences were found between cells treated with oligomycin with or without rotenone, in the presence or absence of ConA. Rotenone alone decreased ATP to the same extent as did oligomycin (results not shown). The release of additional calcium by rotenone is therefore not associated with a decrease in ATP, and is likely to be the result of a decrease in mitochondrial membrane potential.

If the rotenone-releasable calcium represents the exchangeable mitochondrial calcium pool, a rough estimate of total mitochondrial calcium, in nmol/mg of protein, can be made, by using the following assumptions: (1) mitochondria occupy 5% of the total cell volume, as in pig lymphocytes [18]; (2) mitochondrial protein concentration is 1.4 mg/ μl of intramitochondrial water space [18]; (3) about 20% of total mitochondrial calcium is exchangeable in 60 min, as in rat hepatocytes [19], and ConA has no effect on this percentage; (4) all cells in the population give approximately the same response to ConA. From Fig. 2(b), the mitochondrial pool in control cells is 8.6 $\mu\text{mol/l}$ of cell water, or

0.61 nmol/mg of mitochondrial protein; after 3 min of ConA treatment, this pool increases to 23 μ mol/l, or 1.6 nmol/mg of protein. For comparison, the total calcium in rat liver mitochondria before and after 3 min of hormone treatment is 0.83 and 2.23 nmol/mg of protein respectively [9].

It should be possible in principle to estimate the size of the ATP-dependent calcium pool from the calcium released by oligomycin alone as compared with DMSO controls. As shown in Fig. 3, oligomycin decreased ATP to 45% of control values after 5 min, with an additional 5–10% decrease after 20 min (results not shown), indicating a substantial contribution of glycolysis to ATP concentrations in these cells. The glycolytic inhibitor 2-deoxyglucose (50 mM final concn.) was found to decrease ATP to 15% of control values after 20 min; however, no additional calcium was released beyond that released by oligomycin (results not shown). This result may indicate that the ATP-dependent pool is completely released by the relatively modest decrease in ATP with oligomycin alone, although this decrease is evidently not sufficient to inhibit the plasma-membrane ATP-dependent calcium-efflux pathway, since a net calcium efflux was still seen even at 15% of control ATP concentrations. It has been suggested that in other systems (e.g. 2H3 cells [20] and smooth muscle [21]) the plasma-membrane efflux pathway continues to operate at low ATP concentrations; in addition, it may be the increase in ADP which releases endoplasmic-reticulum calcium pools under these conditions [22]. The oligomycin-releasable calcium pool may therefore be the same as the ATP-dependent endoplasmic-reticulum pool. No significant decrease in the size of this pool can be detected after 3 min of ConA treatment: although the size of this pool was significantly smaller in ConA-treated cells after 5 min of oligomycin (Fig. 2), additional calcium was released after 20 min of inhibitor treatment, and no difference between cells with and without ConA was

Fig. 4. Time course of ConA effects on thymocyte calcium

Thymocytes were labelled with ^{45}Ca , ConA was added at zero time, and inhibitors were added after 3, 8, 13 or 18 min; samples were removed 5 min later for determination of intracellular calcium as described in the Experimental section. Inhibitors added were DMSO (Δ), oligomycin ($\square$) and oligomycin + rotenone ($\blacksquare$). Results shown are means $\pm$ S.E.M. for a total of seven to eight determinations from three separate thymocyte preparations.

detected (Fig. 1). This result may indicate either that the endoplasmic-reticulum pool rapidly re-fills after mitogen-induced release, or simply that this method is not sensitive enough to detect a small change in this relatively large pool.

The experiments in Figs. 1 and 2 were designed to detect an intracellular redistribution of calcium after 3 min of ConA treatment. To determine whether the increase in mitochondrial calcium is persistent, cells were pre-treated with ConA for various times before addition of inhibitors for 5 min. As shown in Fig. 4, the exchangeable mitochondrial pool decreased in size rapidly after 3 min and was not detectable after about 10 min of ConA treatment. This result is apparently inconsistent with that obtained by Assimacopoulos-Jeannet *et al.* [9] in which in hormone-treated liver, mitochondrial calcium and dehydrogenase activity continued to increase for more than 10 min. Assimacopoulos-Jeannet *et al.* [9] measured total mitochondrial calcium (rather than exchangeable) and concluded that matrix free Ca^{2+} varies in parallel with total. It is possible that ConA affects exchangeable calcium in a way unrelated to either free or total, although this seems unlikely. The result reported here is consistent with the kinetics of the mitogen-induced changes in cytoplasmic free Ca^{2+} concentrations measured by using the fluorescent indicator quin-2 in mouse thymocytes [23] and some other systems such as mouse 3T3 cells [24]. The rapid rise and fall in the cytoplasmic free Ca^{2+} concentrations in these cells may be paralleled by the mitochondrial calcium pool, as expected if Ca^{2+} transport in mitochondria obeys the same kinetics *in vivo* as *in vitro* [7]. A decrease in mitochondrial calcium has been shown to inactivate dehydrogenases rapidly in rat liver mitochondria [7,25], and therefore any activation of Ca^{2+} sensitive dehydrogenases after mitogenic stimulation may be rapidly reversed as the cytoplasmic Ca^{2+} decreases. It should be noted that the activation of pyruvate dehydrogenase in pig lymphocytes [11] occurs slowly over the course of 2 h, and may therefore be due to a mechanism not directly related to the rapid calcium fluxes.

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