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CAP FORMATION BY VARIOUS LIGANDS ON LYMPHOCYTES SHOWS THE SAME DEPENDENCE ON HIGH CELLULAR ATP LEVELS

T. POZZAN, A.N. CORPS, C. MONTECUCCO *, T.R. HESKETH and J.C. METCALFE

Department of Biochemistry, Tennis Court Road, Cambridge, CB2 1QW (U.K.)

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Summary

The effects of inhibitors of mitochondrial ATP synthesis and the calcium ionophore, A23187, on the capping of surface immunoglobulin, concanavalin A receptors and θ antigen on mouse spleen or thymus cells have been examined.

(i) For all of these capping ligands and inhibitors, the cellular ATP level must be above 80% of the normal level in resting lymphocytes for 90% of maximal cap formation to occur. Below 50% of the normal ATP level, less than 10% of maximal capping occurs. There is, therefore, a common dependence for all three capping systems on the cellular ATP level, irrespective of the metabolic inhibitor used.

(ii) Inhibition of cap formation by A23187 follows the same profile for ATP dependence as the mitochondrial inhibitors, but in contrast to those inhibitors, A23187 requires extracellular calcium to decrease the ATP level and inhibit capping. Other agents can affect cap formation without reducing the ATP level. For example, concanavalin A inhibits its own cap formation and cytochalasin B reduces the rate of cap formation at concentrations which do not alter the cellular ATP level.

(iii) From these and other data we conclude that there are cellular functions essential for cap formation, other than the maintenance of ionic gradients, that require a high concentration of cellular ATP. The possibility that high levels of ATP are required for the function of the cytoskeleton in lymphocytes is discussed.

* Present address: CNR Unit for Physiology of Mitochondria, Istituto di Patologia Generale, Via Loredan, 16, 35100 Padova, Italy.

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Mops, 4-morpholinepropanesulphonic acid; FITC-RaMIg, fluorescein-conjugated rabbit anti-mouse IgG; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; DOPC, dioleoyl phosphatidylcholine.

Introduction

When lymphocytes and other motile cells are incubated with polyvalent ligands, including some lectins and antibodies to surface antigens, a three-phase redistribution of the receptors may occur [1]. The randomly distributed receptors are first cross-linked into discrete patches which are subsequently drawn together to form a cap over the pole of the cell, and finally the cap is ingested or discarded. The formation of the caps, but not the preceding patches, can be blocked by metabolic inhibitors and to a variable extent by cytochalasin B and colchicine, which has been interpreted as evidence for the involvement of microfilaments and microtubules in cap formation by processes dependent on metabolic energy [2—5]. This view has been supported by the demonstrations of concurrent rearrangement of the cytoskeleton by immunofluorescence techniques [6—8], and of the association of cross-linked surface immunoglobulin in the membrane with actin [9]. Various unitary mechanisms for cap formation by different ligands have been proposed [10,11], while other workers have suggested that microfilaments are involved in cap formation by some but not all ligands [7].

In previous work on the effect of ionophores on capping, we have shown that the process was independent of membrane potential and occurred normally in cells either completely depolarised or hyperpolarised with respect to the normal membrane potential of about —62 mV [12,13]. At the same time, it was noted that those ionophores which inhibited capping (e.g., valinomycin) also reduced the cellular ATP level, offering an explanation for their blocking action [13]. Preliminary experiments indicated that relatively small decreases in the total cellular ATP caused a substantial decrease in capping. Here we have examined the effects of a range of metabolic inhibitors which reduce lymphocyte ATP levels on the formation of caps by three different ligands, to determine whether the ATP requirement is the same for different ligands.

Materials and Methods

Fluorescein-conjugated rabbit anti-mouse IgG (FITC-RaMIg) from Miles Yeda was dialysed against Eagle's minimum essential medium (modified) with Earle's salts (Flow Laboratories) buffered at pH 7.3 with 10 mM Hepes (Searle), until centrifugation of the heat-denatured FITC-RaMIg (15 min at 95°C) yielded a supernatant which had no detectable effect on cellular ATP levels or capping by FITC-RaMIg, indicating complete removal of the preservative (mersalyl). AKR anti-Thy 1.2 (anti-θ antibody) was given by Dr. H. Waldmann. Concanavalin A from Miles Yeda was conjugated with fluorescein isothiocyanate (FITC-concanavalin A) as described by Rinderknecht [14] and dialysed against medium as for FITC-RaMIg.

Spleen or thymus cells from 2—3-month-old Balb/c mice were isolated in medium as described previously [12] and suspended at $2—3 \cdot 10^7$ cells per ml. Viabilities determined by eosin exclusion were higher than 95% in all preparations used and were unaffected by any of the experimental procedures described. Aliquots of the cell suspensions were equilibrated at 37°C for 15 min and additions of stock solutions of ligands and/or inhibitor in medium were

made to the final concentrations and for the periods specified. Additions from stock oligomycin solutions (1 mg/ml in 1 : 1 medium : Me_2SO, v/v) and cytochalasin B (Sigma) (1 mg/ml in Me_2SO) were made to give final Me_2SO concentrations of less than 1%, shown to have no effect on FITC-RaMIg capping or cellular ATP levels. When the calcium ionophore, A23187 (gift of Eli Lilly), was added to cell suspensions from Me_2SO or ethanol stock solutions, poor reproducibility in its effects on capping and ATP levels was obtained, particularly when the medium contained low calcium concentrations. Instead, the ionophore (1 mg) was dissolved in $CHCl_3$ with 10 mg of dioleoyl phosphatidylcholine (DOPC), dried under vacuum and resuspended, in 1.0 ml of phosphate-buffered saline containing 2 mM $MgCl_2$, by sonication to homogeneity in a sonicating bath at less than 10°C. The A23187 suspension buffered by DOPC gave reproducible effects on capping and ATP levels and was more potent than additions of the same amounts of A23187 from Me_2SO or ethanol stock solutions. The DOPC vesicles alone had no effect on capping or ATP levels and the effects of ionophore added with DOPC vesicles were rapidly reversible after washing with fresh medium containing 10% bovine serum albumin or 20% fetal calf serum (Flow Labs). The medium for some of the experiments using A23187 was made up without added calcium. The residual 2.5—5 μM calcium contamination (measured by atomic absorption spectroscopy) was buffered by the addition of an equivalent amount of EGTA and titrations of the calcium concentration were made by the addition of $CaCl_2$.

In capping experiments in the presence of inhibitors, the aliquots of cells were usually preincubated with the inhibitor for 5 min before addition of the capping ligand, but for capping with anti-θ plus FITC-RaMIg, the anti-θ was added with the inhibitor 5 min before the addition of FITC-RaMIg. Variations in these protocols are described in the figure legends. Samples of cells for capping measurements were fixed in 1% HCHO in phosphate-buffered saline at 4°C, washed twice in medium at 4°C and examined by ultraviolet fluorescence microscopy (Leitz Diavert microscope). Capping was scored as the percentage of fluorescent cells with caps covering less than 50% of the cell, and the results are expressed as a proportion of the cells capping in control samples treated in the same way without inhibitor, but with the corresponding concentration of Me_2SO where appropriate. 100—200 fluorescent stained cells were counted per sample. Cell samples for ATP assay were treated with 5% perchloric acid at 0°C and neutralised with 0.5 M KOH buffered with 0.1 M Mops. Samples of supernatant (20 μl) were injected into a solution of 10 mg/ml firefly lantern extract (Sigma) in a Du Pont 760 luminescence biometer, calibrated with standard ATP solutions treated in the same way. All ATP results are expressed as a percentage of control cell samples incubated without inhibitor, but with Me_2SO where appropriate.

Results

ATP levels in mouse lymphocytes and the action of metabolic inhibitors

The level of ATP in both spleen and thymus cell preparations was between 550 and 650 amol/cell (600 ± 30 S.D.; 25 preparations), equivalent to an average cellular concentration of 3.0 mM assuming a cell volume of 214 μm^3 [15].

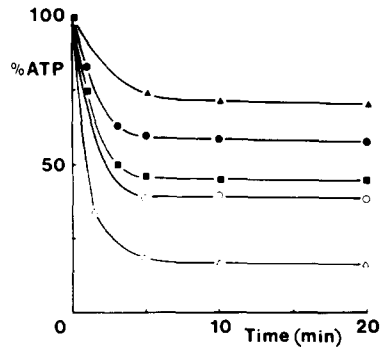

Fig. 1. ATP levels in spleen cells in the presence of mitochondrial inhibitors as a function of time. NaN_3, 0.8 or 5.0 mM (▲, △); 2,4-dinitrophenol, 80 or 150 μM (●, ○); oligomycin 1 μg/ml (■).

When inhibitors of oxidative phosphorylation were added, the ATP levels decreased rapidly over the first 5 min, but very slowly thereafter for at least 30 min (less than 5% drop, see Fig. 1). Only 2-deoxyglucose, which inhibits glucose utilisation, required prolonged incubation (more than 10 min) before a significant decrease in both ATP levels and capping was observed. Depletions of at least 90% of the cellular ATP were substantially reversible by washing the cells in fresh medium containing bovine serum albumin (10 mg/ml) to remove the inhibitors, although no reversal of the effects of oligomycin could be obtained, presumably due to its very high affinity for F_1ATPase (Table I). The results indicate that mitochondria provide the major source of ATP in these cells, in agreement with data for rat thymocytes [16], but in contrast to human peripheral lymphocytes, where mitochondrial inhibitors are relatively ineffective in the presence of glucose [17]. We conclude that the use of mitochondrial inhibitors with mouse lymphocytes provides close control of cellular ATP levels over the time periods required to estimate quantitatively the ATP requirement for capping.

TABLE I

RECOVERY OF CELLS FROM THE EFFECTS OF INHIBITORS OF ATP SYNTHESIS

Cell samples were incubated with inhibitors for 5 min before measuring ATP and initiating capping with FITC-RaMIg as in the legend to Fig. 2a. Parallel cell samples were washed twice in fresh medium containing 10 mg/ml bovine serum albumin after the 5 min incubation with inhibitors, and stood for 15 min at 37° C before measuring ATP and initiating capping. Capping on control cell samples (86% of stained cells) was not significantly affected by the washing procedure. Values are given with standard deviations from four experiments. FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, n.d., not determined.

Preincubation	Before washing		After washing	
	% ATP	% capping	% ATP	% capping
No addition	100	100	100	100
1 μg/ml oligomycin	36 ± 5	3	35 ± 3	1
2 mM NaN_3	41 ± 5	1	68 ± 4	66 ± 4
1 μM rotenone	27 ± 4	0	67 ± 6	65 ± 10
1 μM KCN	32 ± 3	1	96 ± 3	98 ± 2
200 μM 2,4-dinitrophenol	10 ± 1	0	71 ± 7	89 ± 2
0.5 μM FCCP	18 ± 6	0	88 ± 9	95 ± 5
10 mM 2-deoxyglucose	75 ± 5	76	n.d.	n.d.

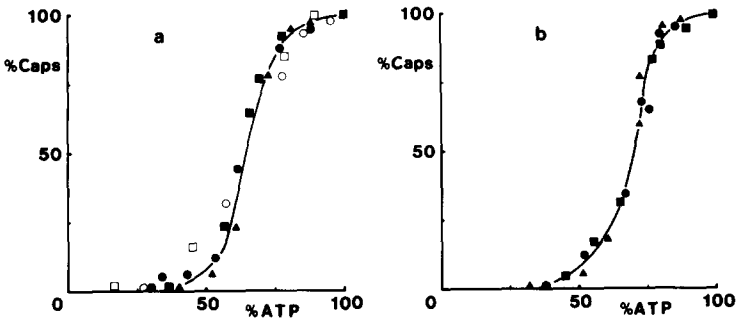

Fig. 2. (a) The relationship between FITC-RaMIg capping and cellular ATP levels. The ATP level was reduced by 5-min preincubations with various concentrations of NaN_3 (▲), 2,4-dinitrophenol (●) and oligomycin (■). Capping was then initiated with 200 μg/ml FITC-RaMIg, and after 7 min the cells were fixed with HCHO. The 100% capping level on control cells was 80—90% of the stained cells. In a separate series of experiments, the effects of A23187 and calcium on cellular ATP levels and capping were compared with the corresponding effects of mitochondrial inhibitors. Reduced ATP levels were obtained by 5-min preincubations with A23187 concentrations from 0.1 to 1.0 μM in normal medium containing 1.8 mM Ca^{2+} (○). Alternatively, the ATP level was reduced by adding 20 μM A23187 to cells in a simplified medium without amino acids or vitamins, containing a range of free calcium concentrations from 0 to 100 μM; in these experiments capping was initiated 30 s after the addition of ionophore (□). (b) Common dependence of cap formation by different ligands on cellular ATP levels. The ATP levels were reduced by a 5 min preincubation with various concentrations of NaN_3. Capping was then initiated on spleen cells with 200 μg/ml FITC-RaMIg (▲) or 10 μg/ml FITC-concanavalin A (●), and on thymocytes (preincubated also with anti-θ at 1 : 600 dilution in medium) with 200 μg/ml FITC-RaMIg (■). Cells were fixed after 7 min (▲), 15 min (●) and 25 min (■), and the 100% level of capping on control cells was 80, 42 and 85% of the stained cells, respectively.

Inhibition of capping by metabolic inhibitors

Spleen cells were preincubated with a range of metabolic inhibitors to reduce ATP levels by up to 90%, to determine the effect on cap formation by FITC-RaMIg. Data for NaN_3, 2,4-dinitrophenol, and oligomycin (Fig. 2a) show that, irrespective of the inhibitor used, there is a sharp decrease in cap formation below about 80% ATP, and less than 10% capping below 50% ATP. Data for the other inhibitors included in Table I are consistent with a common profile for capping as a function of ATP concentration. After washing the cells treated with inhibitors with medium containing 10 mg/ml bovine serum albumin, cap

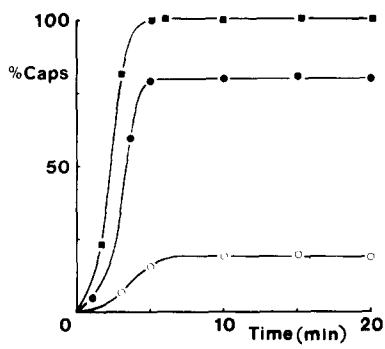

Fig. 3. Rate of FITC-RaMIg capping at different ATP levels. Preincubation with 2,4-dinitrophenol at 70 or 100 μM (●, ○) reduced the ATP level to 70 or 50%, respectively, of the control cells (■). Capping was initiated with 200 μg/ml FITC-RaMIg and the cells were fixed with HCHO at the times indicated.

formation was subsequently restored to an extent determined by the recovery in ATP level (Table I), consistent with the profile in Fig. 2a. The time course of capping was very similar at three different ATP levels in the presence of 2,4-dinitrophenol and longer incubation with FITC-RaMIg did not overcome the block (Fig. 3).

In further experiments, preincubation with NaN_3 was used to compare the dependence on ATP levels of cap formation by FITC-RaMIg and FITC-concanavalin A on spleen cells and anti-θ plus FITC-RaMIg on thymus cells (Fig. 2b). Similar profiles were observed for all three types of cap formation, indicating that the ATP requirement is the same for fast capping (FITC-RaMIg) or slower cap formation by FITC-concanavalin A or anti-θ plus FITC-RaMIg, assayed at 7, 15 and 25 min, respectively (see legend to Fig. 2b). For all ligands and inhibitors, the ATP-capping profile was unaffected by the addition of sufficient EGTA to reduce the free Ca^{2+} level to below 10^{-6} M.

Capping itself had no significant effect on the total ATP levels in the cell populations. Thus, during or after capping with FITC-RaMIg or FITC-concanavalin A on spleen cells, or anti-θ plus FITC-RaMIg on thymocytes (35; 40 and 85% of the total cell population capped, respectively), the ATP level measured after 3—10 minutes was 101 ± 5, 101 ± 4 and 98 ± 5%, respectively.

Inhibition of capping by A23187

It has been shown previously that capping occurs normally without free calcium in the medium, even in the presence of high concentrations of A23187 [18,19]. Thus, the entry of calcium into the cells from the external medium is not involved in the signalling mechanism for cap formation. However, as the calcium concentration is increased in the presence of high concentrations of calcium ionophore, there is a progressive and reversible inhibition of cap formaion, indicating that the entry of calcium into the cells is correlated with the inhibition of capping. In Fig. 2a, the effect on ATP levels of increasing the extracellular calcium concentration in the presence of 30 μM A23187 is shown and compared with the effect on cap formation. It can be seen that the same profile for capping as a function of ATP level is followed by the ionophore as for the inhibitors of mitochondrial ATP synthesis. A very similar profile is also generated by varying the ionophore concentration in the presence of the normal calcium concentration (1.8 mM) in the medium (Fig. 2a). Similar profiles relating capping to ATP levels were also obtained with A23187 for cap formation by both FITC-concanavalin A on spleen cells and anti-θ with FITC-RaMIg on thymocytes (data not shown).

Inhibition of capping by cytochalasin B and concanavalin A

Both cytochalasin B and concanavalin A inhibit cap formation by mechanisms which differ markedly from the metabolic inhibitors in that they do not decrease cellular ATP levels. Cytochalasin B was found to decrease the rate of capping of FITC-RaMIg on spleen cells, FITC-concanavalin A on spleen cells, and anti-θ with FITC-RaMIg on thymocytes (Fig. 4). Maximal capping was eventually achieved in each case by prolonging incubation with the ligand for up to 100 min. The concentration of cytochalasin B used (10 μM) caused no

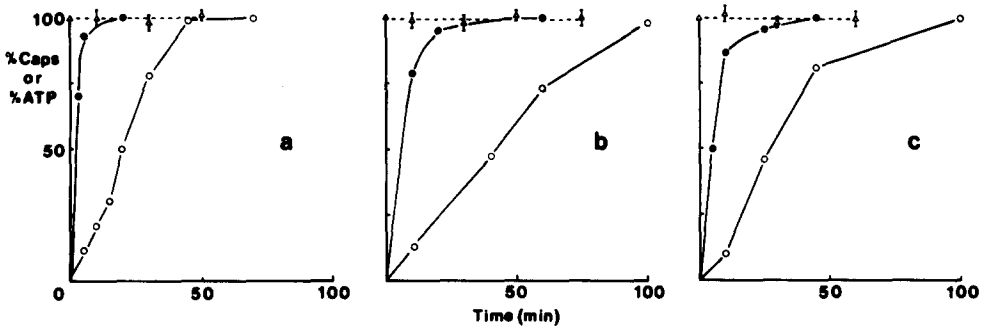

Fig. 4. The effect of cytochalasin B on cellular ATP levels and cap formation. Cytochalasin B (10 μM) was added 5 min before the capping ligand, which was added at time = 0. •, capping of control cells (Me_2SO). ○, capping of cytochalasin B-treated cells. △, ATP levels in cytochalasin B-treated cells. Ligand concentrations and maximal capping were as described in the legend to Fig. 2b. (a) FITC-RaMIg capping on spleen cells. (b) FITC-concanavalin A capping on spleen cells. (c) Anti-θ plus FITC-RaMIg capping on thymocytes

significant change in the ATP levels of either cell type throughout this period (Fig. 4).

As noted previously, FITC-concanavalin A at optimal capping concentrations caused no ATP depletion in spleen cells (102 ± 5% at 10 μg/ml FITC-concanavalin A). At a concentration of 100 μg/ml, where 50% reduction in capping occurred, there was no significant effect of FITC-concanavalin A on the cellular ATP level (101 ± 6%).

Discussion

The effects of the specific inhibitors of oxidative phosphorylation used in this study indicate that most of the ATP in unstimulated murine lymphocytes is of mitochondrial origin. The initial rate of decrease in ATP level at the highest concentration of inhibitor which can be fully reversed is approx. 300 amol/cell per min, which must correspond to the minimum rate at which the cells is consuming ATP at 37°C. This can be compared with an O_2 consumption of 80—110 amol O_2/cell per min, of which 50% is oligomycin sensitive [20]. Assuming an ATP/O ratio of 3, this is equivalent to a rate of mitochondrial ATP synthesis of 240—330 amol/cell per min, consistent with the rate of ATP consumption estimated above.

The inhibition of capping by metabolic inhibitors is well documented. The inhibition of cap formation at below 50% of the normal ATP level is clearly not due to irreversible loss of cellular function or viability, as shown by the recovery in ATP level and cap formation after removing the inhibitors (Table I). The new feature of the present study is the definition of a quantitative relationship between the cellular ATP level and the extent of capping which is the same for all of the ligands studied and independent of the agent used to reduce the ATP level. The unique profile relating the ATP level to cap formation can be used to assess whether an agent which affects cap formation does so through an effect on energy metabolism. For example, over the time period of cap formation, cytochalasin B does not affect the cellular ATP level

significantly (Fig. 4) and any effect it may have on glucose transport into the cells [21] is therefore insignificant in affecting ATP metabolism. However, the effect of cytochalasin B on the kinetics of both fast and slow cap formation is consistent with the involvement of microfilaments in cap formation by all of the ligands used, contrary to some previous evidence [7]. Similarly, the self-inhibition of cap formation by high concentrations of concanavalin A cannot be attributed to metabolic effects resulting in reduced cellular ATP levels. On the other hand, the inhibition of cap formation by valinomycin can be accounted for entirely by its effect on the cellular ATP level and we have shown elsewhere that the inhibition of cap formation does not depend on any effect of valinomycin on the membrane potential [13].

These examples illustrate the diagnostic use of the ATP-capping profile, which is also relevant to the analysis of the effect of the calcium ionophore A23187 on cap formation. The inhibition of cap formation by A23187 does not occur in the absence of external calcium, and under these conditions the ionophore does not reduce the cellular ATP level. We therefore assume that an increased influx of external calcium into the cytoplasm is responsible for the inhibition of capping, and we have shown elsewhere that increases in the cytoplasmic calcium concentration from 1 to 50 μM coincide with a progressive inhibition of cap formation [22]. From the data in Fig. 2a, it is clear that the effect of the ionophore on the cellular ATP level in the presence of external calcium is sufficient to account for the inhibition of capping. This does not preclude the possibility that the increase in cytoplasmic calcium concentration also effects cap formation by mechanisms independent of the effect on ATP level. The ATP-dependent disruption of preformed caps by the ionophore in the presence of external calcium indicates such an effect of calcium on the cytoskeleton, or other cellular functions, involved in maintaining a cap [19]. Our experiments do not eliminate such an effect as the mechanism of inhibition of cap formation, but they do show that the effect on the ATP level is of sufficient magnitude and rate to account, by itself, for the inhibition of capping.

The mechanism by which the ionophore lowers the ATP level in the presence of external calcium is not established. It is known that fast calcium uptake by mitochondria from any tissues inhibits ATP synthesis with a K_m of 1—50 μM [23], and it is also possible that penetration of the ionophore directly to the mitochondria causes rapid calcium cycling, and hence contributes to the ionophore-induced reduction in ATP level.

The requirement for high cellular levels of ATP for cap formation is of considerable interest. It seems very unlikely that the maintenance of ionic gradients of Na^+, K^+ or Ca^{2+} across the plasma membrane by ATP-dependent transport systems is required for cap formation. The inhibition of the $(Na^+ + K^+)$-ATPase by ouabain, or complete depolarisation of the plasma membrane in high $[K^+]$ medium (with or without gramicidin) does not effect cap formation [13], and the ATP-capping profile obtained using metabolic inhibitors is unchanged by the addition of EGTA to the medium to chelate calcium. Another possible explanation of the high levels of ATP required is that capping might itself utilise a significant proportion of the cellular ATP. However, no significant decrease in cellular ATP was observed during cap formation by any

ligand. If capping does use a substantial proportion of cellular ATP, there must be either an immediate and equivalent increase in the rate of cellular ATP synthesis, or a compensating decrease in ATP consumption by other ATP-dependent systems. Neither of these possibilities seems likely. We are investigating the possibility that a high ATP level is required for the maintenance of a dynamic equilibrium in the structure of the cytoskeleton in the resting cell. If this is correct, a drop in ATP level might be expected to inhibit cap formation by all ligands, assuming that they all cause cap formation by a common mechanism involving the cytoskeleton.

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