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TRIGGERING OF LYMPHOCYTE CAPPING APPEARS NOT TO REQUIRE CHANGES IN POTENTIAL OR ION FLUXES ACROSS THE PLASMA MEMBRANE

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Summary

Capping induced by anti-Ig antibody on mouse spleen lymphocytes was found to proceed normally over a wide range of membrane potentials from approx. 0 to —65 mV, as estimated with fluorescent probes. The potential was manipulated by ionic substitution in the medium and/or application of gramicidin.

Various agents which inhibit capping had differing effects on the membrane potential, some producing no measurable change, others depolarising the cells. In particular valinomycin (10^{-7} M) was found to inhibit capping in cells both slightly hyperpolarised from the normal resting potential, and fully depolarised. Valinomycin was found to deplete the lymphocytes markedly of ATP and this effect was sufficient to account for the inhibition of capping.

Capping occurred in a simplified (sucrose) medium lacking Na^+, K^+ and Ca^{2+}, suggesting that fluxes across the plasma membrane of these ions are not required.

It is concluded that after ligand binding, some reorganisation of receptor protein at the inner face of the membrane is the sufficient stimulus for the intracellular rearrangements involved in capping.

Introduction

Lymphocyte capping is a well defined response to high concentrations of various surface receptor ligands. In B cells, binding of anti-Ig antibody to sur-

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Abbreviations: diS-C_3-(5), 3,3′-dipropylthiadicarbocyanine; bis-oxonol, bis-(1,3-diethyl-thiobarbiturate)-trimethineoxonol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-3-propanesulfonic acid.

face Ig induces a three phase reorganisation of receptors. The dispersed receptors first gather into randomly distributed patches. This process seems to depend only on crosslinking of the receptors by the divalent antibody and occurs in metabolically poisoned cells, or at 4°C. Next the patches aggregate into a cap and finally the cap is 'internalised' or shed into the medium [1,2]. The mechanisms controlling cap formation are not well understood though many observations implicate microfilaments and microtubules in collecting surface receptors at the pole of the cell (see e.g. Refs. 3—5). Rather little attention has been paid to the way in which ligand binding may trigger this cytoskeletal reorganisation. One suggestion is that changes in membrane potential and ion fluxes could be involved, as the case in many other instances of stimulus-activity coupling. Daniele and Holian [6] found that the K^+ ionophore, valinomycin, blocked cap formation in human B cells, and proposed that the valinomycin was acting by maintaining a hyperpolarisation of the membrane. In the previous paper [7] we have shown that lymphocyte membrane potential can be assessed with fluorescent probes and that valinomycin does somewhat hyperpolarise lymphocytes suspended in standard media. The present work explored the effects of alteration of membrane potential induced by ionophores, changed ionic environment and other agents, on the initiation of capping in mouse spleen B cells.

Methods

Mixed lymphocyte and B-enriched suspensions were prepared from the spleens of BALB/c and nu/nu mice as described in the previous paper. Also described there are the procedures for assessing membrane potential with the fluorescent probes dipropylthiadicarbocyanine (diS-C_3-(5)), and bis(1,3-diethylbarbiturate)-trimethineoxonol (bis-oxonol), and the measurement of capping responses, and also the sources of materials. Some capping experiments were done in the modified Hank's medium (Hepes buffered) used for cell isolation, with some $3 \cdot 10^7$ cells/ml. Many experiments were done with cells suspended in the simplified solutions used for assessment of potential. The standard solution contained 139 mM NaCl, 6 mM KCl, 0.8 mM $MgCl_2$, 1.2 mM $CaCl_2$, 1 mM $NaHPO_4$, 5 mM Na^+-Hepes, 10 mM dextrose, pH 7.2 at 37°C. In high K^+ and choline solution KCl or choline chloride isotonically replaced NaCl. The usual protocol for capping experiments was to incubate a 50 μl aliquot of lymphocyte suspension for 5 min at 37°C and then add any agent to be tested for inhibitory effect. Hydrophobic agents such as gramicidin and valinomycin were added from Me_2SO stock and the controls then had Me_2SO added. After 5 min exposure to the test agent 4 μl of Miles-Yeda fluorescein-labelled rabbit anti-mouse Ig antibody was added and the suspension incubated for five more minutes at 37°C. The cells were then fixed and assessed for staining and capping in the usual way. At least 200 stained cells were counted in each test and the number capped expressed as a percentage of the total number of stained cells. Viability in all cases, even after exposure to inhibitors, was more than 95%, assessed by eosin red exclusion.

Results

Effects of valinomycin on mouse B cell capping

Valinomycin inhibited anti-Ig antibody induced capping in Hank's medium or in standard physiological saline. 50% inhibition was seen with less than 10^{-8} M valinomycin and almost complete inhibition was produced by 10^{-7} M valinomycin. These results are very similar to those reported for human B cells [6]. Next the effects of valinomycin in high K^+ solutions was examined. In 145 mM KCl medium the inhibitory effect of valinomycin on capping was the same as that seen in normal medium. High K^+ solutions depolarise lymphocytes [7,8] and valinomycin produces a slight further depolarisation [7]. These results seem to rule out the hyperpolarising action of valinomycin, in normal K^+ solution, as the basis of its inhibitory action. It was previously reported that while isotonic replacement of 50 mM NaCl with KCl did not affect the inhibitory effect of 10^{-7} M valinomycin, addition of 50 mM KCl to standard medium abolished these effects in human B cells [6]. We have been unable to find any such effect in mouse cells. As shown in Table I, where much of the data in this paper is summarised, addition of 50 mM KCl to standard medium failed to prevent the fully inhibitory effect of 10^{-7} M valinomycin.

TABLE I

CAPPING AND MEMBRANE POTENTIAL IN MOUSE SPLEEN LYMPHOCYTES IN DIFFERENT SOLUTIONS AND AFTER ADDITION OF DIFFERENT AGENTS

Except for the modified Hank's medium the solutions were based on the normal physiological saline described in Methods section. In the high K^+ and choline solutions, KCl or choline chloride replaced NaCl isotonically. In the normal + 50 mM KCl solution solid KCl was added to increase the KCl concentration by 50 mM. The simplified sucrose solution contained 260 mM sucrose, 1 mM $MgCl_2$ and 2 mM Tris-Hepes. The membrane potentials are estimated from fluorescent probe data from the previous paper [7] or from experiments described in the text. In the B cell preparation tested here 90% of the cells stained with antibody. Val, valinomycin; Gram, gramicidin; Con A, concanavalin A; CPZ, chlorpromazine.

Cells	Medium	Agent added	Membrane potential (mV)	% of stained cells capping
Mixed	Hank's	—	−62	87
	Normal	—	−62	89
	Normal + 50 mM KCl	—	−35	80
	Choline chloride	—	−65	79
	Simplified sucrose	—	−60	81
	Normal	10^{-7} M Val	−80	1
	High K^+	10^{-7} M Val	+2	2
	Normal + 50 mM KCl	10^{-7} M Val	−30	2
	Normal	10^{-6} M Gram	0	90
	High K^+	10^{-6} M Gram	0	87
	Choline	10^{-6} M Gram	−65	81
	Hank's	50 μg/ml Con A	−60	1
	Hank's	10^{-6} M A23187	−20	3
	Hank's	$2 \cdot 10^{-4}$ M CPZ	−30	4
	Hank's	1 μg/ml Oligomycin	−60	2
B cells from nu/nu mice	Normal	—	−55	85
	High K^+	—	0	80
	Choline	—	−60	75
	Normal	10^{-6} M Gram	0	81

Effects of alteration of membrane potential by high K^+ and gramicidin

Capping proceeded normally in an isotonic K^+ solution, although this depolarises the cells. In standard solution, gramicidin, which confers a large Na^+ as well as K^+ permeability, depolarises lymphocytes [7], but addition of 10^{-6} M gramicidin to the suspension prior to application of antibody did not detectably alter the extent of capping. Nor was gramicidin found to modify the rate at which capping occurred, the process being completed in 5 min at 37°C in the presence and absence of the ionophore. Furthermore the time course of capping, at 20°C, was not affected by 10^{-6} M gramicidin. That the gramicidin had fully depolarised these lymphocytes was confirmed by taking an aliquot of the treated cell suspension and examining the cells with the fluorescent probes diS-C_5-(5) and bis-oxonol.

Effects of conditions which should prevent depolarisation

Next, capping was examined under conditions in which membrane depolarisation in response to physiological stimuli is very unlikely to occur. First choline, a large impermeant cation, replaced Na^+, a substitution which does not itself much alter membrane potential [7] but which should prevent any depolarisation resulting from an increase in Na^+ permeability. Capping however was virtually normal in the choline solution. Addition of gramicidin in choline medium which usually slightly hyperpolarised the lymphocytes [7] and should strongly maintain the hyperpolarised state (acting as a kind of chemical voltage clamp, with the large outward gradient of K^+ and Na^+) also failed to inhibit the capping response.

To see whether any role for external ions in the initiation of capping could be supported cells were incubated in a medium containing only 260 mM sucrose, 1 mM $MgCl_2$ and 2 mM Tris-Hepes. Capping still occurred in 79% of the stained cells.

Effects of inhibitors of capping on membrane potential

DiS-C_3-(5) was used to follow changes in membrane potential of lymphocytes in standard medium after addition of various agents, other than valinomycin, known to block B cell capping. Concanavalin A had no measurable effect on the membrane potential. Chlorpromazine slowly depolarised the cells over a five minute period. A23187, in the presence of Ca^{2+}, likewise depolarised the cells, probably by dissipating the K^+ gradient [7]. The known mitochondrial blockers, oligomycin and rotenone had no effect on the membrane potential at doses which blocked capping. This was not, however, surprising since diS-C_3-(5) itself blocks capping apparently by mitochondrial blockade [9]. The reasons for supposing that diS-C_3-(5) gives a reliable indication of the membrane potential, despite its side effects, include data obtained with the non-toxic probe bis-oxonol [7] and are fully discussed in the preceeding paper. Here again there is no direct correlation between membrane potential and inhibition of capping.

Why does valinomycin inhibit capping?

Daniele and Holian [6] considered the idea that valinomycin might be acting as a metabolic poison uncoupling mitochondria, and since our evidence makes a

role for changes in membrane potential unlikely we re-examined the notion of metabolic blockade. ATP levels in lymphocytes suspended in Hank's medium were measured and the effects of 10^{-7} M valinomycin and $2 \cdot 10^{-6}$ M FCCP (a known potent uncoupler of mitochondria and inhibitor of capping [10]) were observed. Valinomycin produced an 87% reduction in ATP and FCCP a 90% reduction compared with untreated control cells. Valinomycin produced a similar ATP depletion in isotonic KCl medium. 10^{-6} M gramicidin by contrast, which did not inhibit capping, had no measurable effect (<10% depletion) on cellular ATP levels. It appears that valinomycin acts as a potent metabolic poison in these cells.

Discussion

These results show that capping can occur in mouse spleen B cells in a variety of conditions which result in widely different membrane potentials from approx. —65 to 0 mV. Conversely, inhibitors of capping had disparate effects of membrane potential, concanavalin A and oligomycin, for example, evoking no change in potential, chlorpromazine and A23187 producing depolarisation. Inhibition of capping by valinomycin did not seem to be related to changes in membrane potential since this inhibition was seen in 6 mM K^+ solutions where the ionophore slightly hyperpolarises the cells and in isotonic K^+ solutions where it causes a small further depolarisation in the already largely depolarised cells. The inhibitory action of valinomycin seems to be well explained by its ability to uncouple mitochondria and deplete the cells of 87% of their ATP. (The fact that gramicidin does not inhibit capping, nor deplete cellular ATP, despite its ability to uncouple isolated mitochondria suggests that this antibiotic does not enter the cytoplasm in significant amounts when applied to cell suspensions, but presumably partitions very strongly into the plasma membrane.)

The maintainance of capping in solutions where choline chloride or sucrose replaced NaCl strongly suggests that an inward movement of Na^+ is not involved in triggering the process. Since external Ca^{2+} is also unnecessary for capping [1] it is difficult to conceive what source of inward current (for depolarisation) might be available to the cells. A requirement for any membrane depolarisation is made even less likely by the virtually normal capping seen in choline solution in the presence of gramicidin where the membrane potential should be under firm 'chemical voltage-clamp.' The ability of B cells to cap in a medium containing only sucrose, $MgCl_2$ and Tris-Hepes makes any role for a flux of Na^+, K^+, or Ca^{2+} across the plasma membrane difficult to support. A further point of interest is the failure of treatment with gramicidin in standard medium to alter the extent or rate of capping, since not only will the cells be depolarised by they also will substantially exchange their internal K^+ for Na^+. It seems that the intracellular monovalent cation environment may not be critical for successful capping.

Despite the apparent indifference of the cells to imposed alterations of membrane potential, in terms of triggering capping, it remains a possibility that some change in potential could occur as a consequence of the capping process. Our preliminary experiments with the fluorescent probes indicate that any such

change is small, perhaps a few millivolts of depolarization following anti-Ig antibody-induced capping. But this result will require confirmation with purified B cell suspensions for quantitative resolution of this point.

Changes in potential and ion fluxes across the plasma membrane are unlikely to be triggers for capping. We therefore conclude that some reorganisation of receptor protein at the internal face of the membrane, following cross linking by multivalent ligand, is a sufficient stimulus to the intracellular rearrangements involved.

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