Kinetic evidence for a common mechanism of capping on lymphocytes

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(Received 14 October 1981/Accepted 31 December 1981)

1. Differences in the rates at which ligands cap various receptors on the same cells, and their sensitivity to various drugs, have been interpreted as evidence that there are distinct mechanisms for 'fast' and 'slow' cap formation. We have examined the factors which determine the rate of cap formation of three receptors on mouse splenic lymphocytes or thymocytes, and compared the effects of cytochalasin B or colchicine under conditions where the different receptors cap at similar rates. 2. When surface immunoglobulin, concanavalin A receptors, or θ antigen are induced to cap at their maximal rates by appropriate concentrations of one or more cross-linking ligands, the half-time for maximal capping of each receptor population is between 1.5 and 3.0 min at 37°C. Slower rates of cap formation are obtained by using non-optimal concentrations of the cross-linking ligands. 3. When the three receptors were induced to cap at similar rates (either maximal or slower), 10 μ M-cytochalasin B caused a similar decrease in the rate of cap formation for each receptor, without affecting the eventual extent of capping. At comparable capping rates on control cells, colchicine (10 μ M) increased the rate of cap formation for surface immunoglobulin and concanavalin A receptors to a similar extent, without affecting the eventual extent of cap formation. In contrast, colchicine had no detectable effect on the capping of θ antigen. 4. From these results, we conclude that there are no intrinsic differences in the rates at which different receptors can be induced to cap that can be used to diagnose differences in their mechanisms of cap formation. The observation that ligand concentration and the drugs acting on the cytoskeleton generally affect the rate but not the extent of cap formation accounts for the wide variation in reported effects of the drugs on cap formation measured at fixed times. The receptor-specific effect of colchicine on surface immunoglobulin and concanavalin A receptors, but not θ antigen, is not readily compatible with models of cap formation which depend on lipid or membrane flow.

It has been proposed that complexes of cross-linking ligands with their receptors on cell surfaces can be divided into two classes distinguished by the rate at which they cap (Braun *et al.*, 1978*a,b*). 'Fast capping', for example of sIg by anti-immunoglobulin antibody, was found to be accompanied by the redistribution of myosin to form a cytoplasmic sub-cap (Braun *et al.*, 1978*a*), and to be sensitive to inhibition and reversal by A23187 or chlorpromazine (Braun *et al.*, 1978*b*). 'Slow' capping, for example of Thy 1.2 (θ) antigen by two layers of

cross-linking antibodies, did not show redistribution of myosin and was less sensitive to drugs which affected fast capping. The conclusion was drawn that the mechanisms of fast and slow capping could be distinguished by the involvement of cytoskeletal elements in fast, but not slow cap formation.

However, the experimental basis of this distinction is challenged by opposing evidence at several points. Hoessli *et al.*, (1980) found that sIg and θ antigen were capped at similar rates, and that caps of either receptor were associated with sub-caps of cytoskeletal α -actinin. Similarly Singer and colleagues found redistribution of cytoskeletal proteins for all ligands studied (Ash *et al.*, 1977; Bourguignon & Singer, 1977). Similar experimental discrepancies also exist in the reported effects of drugs assumed to act on the cytoskeleton, on capping by sIg and θ

Abbreviations used: sIg, surface immunoglobulin; Ig, immunoglobulin; FITC, fluorescein isothiocyanate; RITC, rhodamine isothiocyanate.

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antigen. For example, rather variable inhibition of sIg capping by cytochalasin B has been reported (De Petris, 1975; Edelman *et al.*, 1973; Loor & Angman, 1980; Schreiner & Unanue, 1976; Taylor *et al.*, 1971; Unanue *et al.*, 1974), and while one report has indicated no effect of this drug on θ antigen capping (De Petris, 1975), we found a reduction in rate, but not extent, of both θ antigen and sIg capping (Pozzan *et al.*, 1980). We also found (Pozzan *et al.*, 1980) that ionophore A23187 inhibited capping of θ antigen, in apparent contrast to the work of Braun *et al.* (1978b).

We would predict that if cap formation occurs by the same mechanism for all receptors, and if they are present at similar concentrations in the membrane, it should be possible to drive them all to cap at similar maximum rates by optimizing the rate of the cross-linking reaction. Under these conditions, a common mechanism of capping for different receptors would require that their responses to drugs, including those acting on the cytoskeleton, should be similar.

Here we have tested these predictions by re-examining the factors which determine the rate of capping of three different receptor populations on mouse splenic lymphocytes and thymocytes, and the effects of cytochalasin B and colchicine. We suggest that many of the apparent contradictions in previous reports can be resolved by taking the rate at which capping occurs into account, and we consider the results in relation to the two main models which have been proposed for cap formation (Bourguignon & Singer, 1977; Bretscher, 1976).

Experimental

Spleen cells and thymocytes were isolated from 2–3 month Balb/c mice as previously described (Hesketh *et al.*, 1977), and resuspended at between 0.5×10^7 and 1.0×10^7 cells $\cdot$ ml $^{-1}$ in minimal essential medium (Earle's salts), buffered with 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] at pH 7.3. Long-term (>1 h) incubations were carried out using RPMI 1640 medium buffered with 20 mM-Tris/bicarbonate in a gassed incubator with an atmosphere of air/CO $_2$ (19:1). Concanavalin A, FITC-rabbit anti-mouse IgG (whole molecule) and anti-concanavalin A antibody were obtained from Miles Yeda, and RITC-concanavalin A was prepared by the method of Rinderknecht (1962). Anti-Thy 1.2 (anti- θ) antibody was a monoclonal IgM from Olac. All ligands were dialysed extensively against RPMI 1640 to remove preservatives before use. Colchicine (Sigma) was made up as a 2 mM stock solution in RPMI 1640, and cytochalasin B (Sigma) stock solutions were in dimethyl sulphoxide (final concn. less than 0.5%).

Aliquots of the cell preparations were pre-

incubated for 30 min at 37°C before addition of ligand. Colchicine was added for the whole of the preincubation period, and cytochalasin B (or dimethyl sulphoxide in controls) was added for the last 5 min of the preincubation. In experiments with concanavalin A receptors or θ antigen on thymocytes for which two crosslinking ligands were used, the primary ligand (concanavalin A or anti- θ antibody) was added for the 30 min preincubation, before washing the cells by centrifugation (3 s, 14 000 g) and resuspending them in fresh medium containing the appropriate concentration of the second ligand (anti-concanavalin A antibody or FITC-rabbit anti-mouse IgG), with or without cytochalasin or colchicine.

Capping was stopped by the addition of an equal volume of ice-cold 2% formaldehyde in phosphate-buffered saline. In the long-term experiments performed using Tris-buffered RPMI medium, this protocol was found to give formaldehyde-induced binding of fluorescent ligand to the cells. This binding was avoided by stopping cap formation by the addition of 10 vol. of ice-cold phosphate-buffered saline, then washing the cells by centrifugation and resuspension in 1 vol. of ice-cold phosphate-buffered saline before the addition of formaldehyde. This protocol gave the same estimates of cap formation when used for short-term experiments as that described above. The cells were examined under u.v. illumination in a Leitz Diavert microscope: 100–200 stained cells were counted per sample, and capping was scored as the percentage of these cells on which fluorescence was restricted to less than half of the cell (Schreiner & Unanue, 1976). From the data thus obtained, the time for half-maximal capping was estimated, and rates of capping are expressed as the reciprocal of this time (Table 1, Figs. 5 and 6). We emphasize that the use of the term 'rate of capping' is not intended to imply a uniform rate of movement of ligand-receptor complexes on each cell, but rather the average rate for a population of cells that we assume to be heterogeneous in this respect.

It should be noted that significant differences in the rates of capping have been observed with different preparations of fluorescent ligand, particularly RITC-concanavalin A [e.g., compare the data in the present paper in Figs. 2 and 4 and Table 1 with previous data in Pozzan *et al.* (1981)]. However, we also found that similar capping rates are observed at optimal concentrations of fluorescent ligand, irrespective of the preparation (Pozzan *et al.*, 1981).

Results

Effect of ligand concentration

The effect of increasing FITC-rabbit anti-mouse IgG concentration on the extent of cap formation

after 7 min incubation at 37°C is shown in Fig. 1(a). The data are very similar those described previously for capping by FITC-rabbit anti-mouse IgG on mouse lymphocytes at an arbitrary fixed time (Taylor *et al.*, 1971). At concentrations below 40 $\mu\text{g} \cdot \text{ml}^{-1}$ there is very little capping, and above 200 $\mu\text{g} \cdot \text{ml}^{-1}$ capping is maximal, although a reduction in the extent of capping was sometimes observed at very high ligand concentrations ($>1 \text{ mg} \cdot \text{ml}^{-1}$). A more revealing pattern of response was observed when the rate of cap formation was measured at increasing FITC-rabbit anti-mouse IgG concentrations. Fig. 1(b) shows that the rate of capping increased with the concentration of FITC-rabbit

anti-mouse IgG even above the levels apparently optimal for capping in Fig. 1(a) ($>200 \mu\text{g} \cdot \text{ml}^{-1}$), and that maximal capping could be obtained at all antibody concentrations indicated, if the incubation was sufficiently prolonged. We conclude that increasing the ligand concentration increased the rate but not the extent of cap formation. Fig. 2 shows that the rate of capping of concanavalin A receptors on spleen Ig⁺ cells depends on ligand concentration in a similar way to FITC-rabbit anti-mouse IgG capping.

The rate of capping of θ antigen on thymocytes by a fixed concentration of anti- θ antibody, followed by a second layer of FITC-rabbit anti-mouse IgG also increases with the FITC-rabbit anti-mouse IgG concentration (Fig. 3a). However, the overall control of the capping rate of θ antigen is more complicated, in that increasing the concentration of anti- θ antibody in the first labelling layer decreases the rate of capping by a fixed FITC-rabbit anti-mouse IgG concentration in the second layer (compare 400 $\mu\text{g} \cdot \text{ml}^{-1}$ FITC-rabbit anti-mouse IgG capping in Figs. 3a and 3b). At relatively high anti- θ antibody concentrations, the extent of capping may also be reduced, particularly at low FITC-rabbit anti-mouse IgG concentrations (Fig. 3b). Fluorimetric measurements showed that the amount of FITC-rabbit anti-mouse IgG bound to the cells increased with the higher anti- θ antibody con-

Fig. 1. Dependence of FITC-rabbit anti-mouse IgG cap formation on ligand concentration

Spleen cells were preincubated for 30 min at 37°C before the addition of FITC-rabbit anti-mouse IgG at the indicated concentrations. (a) Extent of cap formation at different FITC-rabbit anti-mouse IgG concentrations after 7 min. (b) Rate of cap formation at different FITC-rabbit anti-mouse IgG concentrations ($\mu\text{g} \cdot \text{ml}^{-1}$).

Fig. 2. Rate of cap formation on spleen Ig⁺ cells at different RITC-ConA concentrations

Spleen cells were preincubated for 30 min at 37°C before addition of RITC-concanavalin A at the indicated concentrations ($\mu\text{g} \cdot \text{ml}^{-1}$). After fixing at the times shown, the cells were washed with ice-cold phosphate-buffered saline, incubated with FITC-rabbit anti-mouse IgG (200 $\mu\text{g} \cdot \text{ml}^{-1}$) for 5 min on ice, and washed again before examination. The percentage of Ig⁺ cells bearing RITC-concanavalin A caps was then estimated.

Fig. 3. Dependence of the rate and extent of anti- θ plus FITC-rabbit anti-mouse IgG capping on thymocytes on both ligand concentrations

Thymocytes were preincubated with anti- θ for 30 min at 37°C , and then washed into fresh medium containing FITC-rabbit anti-mouse IgG at the indicated concentrations ($\mu\text{g}\cdot\text{ml}^{-1}$). (a) Anti- θ dilution 1:10000; (b) anti- θ dilution 1:5000.

centrations, and we think it likely that the observed decreases in rate and extent of capping reflect the balance between intra- and intermolecular cross-linking at either (or both) layers of antibody binding (see the Discussion).

The capping of θ antigen or concanavalin A receptors on splenic T cells or thymocytes has generally been considered to require a second layer of cross-linking ligand (De Petris, 1975; Loor, 1974; Taylor *et al.*, 1971), as for example in the experiments in Fig. 3. However, the data in Fig. 4 show that the receptors cap with each primary ligand with a half-time of hours rather than minutes. Increasing the concentration of either primary ligand alone increased the rate of capping (results not shown), but

Fig. 4. Acceleration of capping on thymocytes by the addition of a second ligand

Thymocytes were incubated with the first ligand, and at the time indicated ($\downarrow$) the cells were washed into fresh medium containing the second ligand (or equivalent volume of medium). (a) RITC-concanavalin A ($1\mu\text{g}\cdot\text{ml}^{-1}$) with ($\circ$) and without ($\bullet$) anti-concanavalin A ($50\mu\text{g}\cdot\text{ml}^{-1}$). (b) Anti- θ (1:5000 dilution) with ($\square$) and without ($\blacksquare$) FITC-rabbit anti-mouse IgG ($250\mu\text{g}\cdot\text{ml}^{-1}$). To visualize capping by anti- θ alone (b) capping was stopped at the times shown by the addition of 10 vol. of ice-cold phosphate-buffered, then unbound anti- θ was removed by centrifuging the cells (3 s, $14000g$) and resuspending in fresh ice-cold phosphate-buffered with FITC-rabbit anti-mouse IgG ($250\mu\text{g}\cdot\text{ml}^{-1}$). After 5 min incubation on ice, the cells were fixed with formaldehyde and washed before examination.

at concanavalin A concentrations over $5\mu\text{g}\cdot\text{ml}^{-1}$, there are toxic effects on thymocytes within the timescale of cap formation. The effect of the second layer of cross-linking for each receptor is simply to accelerate the rate of cap formation, as shown in Fig. 4. The increase in rate due to the addition of the second ligand does not result from increased binding

of the primary ligand, since unbound primary ligand is removed by washing at the end of the pre-incubation before addition of the second ligand (see legend and the Experimental section). In general, as the concentration of the primary ligand is increased, the concentration of secondary ligand required to drive rapid cap formation also increases.

Effect of cytochalasin B

The effect of cytochalasin B concentrations up to $10\mu\text{M}$ on the rate of cap formation by a fixed FITC-rabbit anti-mouse IgG concentration is shown in Fig. 5(a). In all experiments, the drug reduced the rate but not the final extent of capping. Similar results were also obtained at different FITC-rabbit anti-mouse IgG concentrations as summarized in Fig. 5(b), where the rates of capping are indicated by the reciprocal of the time required for half-maximal capping to occur. It can also be seen from Fig. 5(b) that a fixed concentration of cytochalasin B has a relatively larger effect on the slower cap formation which occurs at lower ligand concentrations. The data indicate that the rate-decreasing effect of cytochalasin B can be titrated against the rate-increasing effect of the ligand concentration to produce a constant rate of capping.

The reduction in the rate, but not extent, of capping by cytochalasin B was shown to apply to all ligand-receptor systems studied (Table 1), including the slow capping of anti- θ antibody or concanavalin A on thymocytes. In all cases the magnitude of the reduction in rate depended directly on the concentration of cytochalasin, but decreased with increasing ligand concentration (results not shown).

In particular, if capping by different ligand systems was adjusted to similar rates, then a given cytochalasin concentration caused a similar reduction in rate (see Table 1).

Effects of colchicine

In Fig. 6 we show that colchicine increases the rate but not the extent of capping by FITC-rabbit anti-mouse IgG at all colchicine and ligand concentrations indicated. This effect of colchicine on sIg capping has not been reported previously, but we note that small increases in the extent of capping at a fixed time were observed (Edelman *et al.*, 1973; Taylor *et al.*, 1971), and we presume that a much larger effect would have been observed at an earlier time.

Colchicine also increases the rate of capping by concanavalin A $\pm$ anti-concanavalin A antibody, either as a fast or slow capping process on spleen cells or thymocytes (Table 1). However, it has no effect on the capping of θ antigen either by anti- θ antibody alone (slow capping) or by anti- θ antibody with FITC-rabbit anti-mouse IgG (fast capping). This lack of effect of colchicine on θ antigen capping is apparently receptor-specific, since, as noted above, capping of concanavalin A ($\pm$ anti-concanavalin A antibody) on the same cells is increased in rate by colchicine.

Combined drug effects

We would predict from the above data that if cytochalasin B and colchicine act independently on the rate of cap formation, then their combined effects should be antagonistic. This prediction is

Table 1. *Effects of cytochalasin B and colchicine on the rate of capping by different ligands*

Experimental protocols were as described in the Experimental section and in the Figure legends. (a) For the short-term capping experiments, the cytochalasin B and colchicine concentrations were each $10\mu\text{M}$; the times for half-maximal capping are given to the nearest half min. (b) For the long-term capping experiments, the cytochalasin B concentration was $1\mu\text{M}$ and the colchicine concentration was $1.5\mu\text{M}$; the times for half-maximal capping are given to the nearest half h. Abbreviations used: Con A, concanavalin A; RAMIg, rabbit anti-mouse immunoglobulin G.

Cell types	Ligands ($\mu\text{g} \cdot \text{ml}^{-1}$)	Time for half-maximal capping		
		Control	Cytochalasin B	Colchicine
(a) Short-term capping			(min)	
Spleen Ig ⁺	FITC-RaMIg (100)	5.5	35	2.0
Spleen Ig ⁺	RITC-Con A (50)	6.5	40	3.0
Thymocyte	RITC-Con A (1) + anti-Con A (50)	4.0	20	2.0
Thymocyte	Anti- θ (1 : 5000) + FITC-RaMIg (250)	5.0	25	5.5
(b) Long-term capping			(h)	
Spleen Ig ⁺	RITC-Con A (1)	2.5	5.0	1.0
Spleen Ig ⁻	RITC-Con A (1)	6.0	11.0	3.0
Thymocyte	RITC-Con A (1)	3.5	9.0	1.5
Thymocyte	Anti- θ (1 : 2000)	5.5	11.0	5.5

Fig. 5. Reduction by cytochalasin B of the rate of cap formation by FITC-rabbit anti-mouse IgG

Spleen cells were preincubated for 30 min at 37°C, and cytochalasin B was added 5 min before the end of the preincubation. FITC-rabbit anti-mouse IgG was then added at the indicated concentrations. (a) Effect of increasing cytochalasin B concentration (μM) on capping by FITC-rabbit anti-mouse IgG ($200 \mu\text{g} \cdot \text{ml}^{-1}$). (b) Reduction by cytochalasin B of the rate of capping (expressed as the reciprocal of the time required for half-maximal capping to be attained) by different FITC-rabbit anti-mouse IgG concentrations ($\mu\text{g} \cdot \text{ml}^{-1}$).

Fig. 6. Increase by colchicine of the rate of cap formation by FITC-rabbit anti-mouse IgG

Spleen cells were preincubated with colchicine for 30 min at 37°C, and FITC-rabbit anti-mouse IgG was then added at the indicated concentrations. (a) Effect of increasing colchicine concentration (μM) on capping by FITC-rabbit anti-mouse IgG ($100 \mu\text{g} \cdot \text{ml}^{-1}$). (b) Increase by colchicine of the rate of capping (defined as in the legend to Fig. 5) by different FITC-rabbit anti-mouse IgG concentrations ($\mu\text{g} \cdot \text{ml}^{-1}$).

confirmed by the data in Fig. 7 for FITC-rabbit anti-mouse IgG capping, which also show that the final extent of cap formation is unaffected. Furthermore, colchicine had no effect on the rate of θ antigen cap formation in the presence of cytochalasin, again consistent with their separate effects on capping of θ antigen. However, we find that if much higher concentrations of the drugs are used

(100 μM colchicine or vinblastine; 20 μM cytochalasin B) and the preincubation time is increased to 2 h, for comparison with previous studies (De Petris, 1975; Edelman *et al.*, 1973; Stern & Bretscher, 1979; Taylor *et al.*, 1971), then the two drugs act synergistically in reducing the rate not the extent of cap formation. Under these conditions, measure-

Fig. 7. Combined effects of cytochalasin B and colchicine on cap formation by FITC-rabbit anti-mouse IgG. Spleen cells were preincubated with $10\mu\text{M}$ -colchicine for 30 min at 37°C , and $10\mu\text{M}$ -cytochalasin B was added 5 min before the end of the preincubation. FITC-rabbit anti-mouse IgG ($250\mu\text{g}\cdot\text{ml}^{-1}$) was then added and the samples were fixed after the times shown. (■), control; (●), cytochalasin B only; (○), colchicine only; (◐) colchicine plus cytochalasin B.

ment of capping at an appropriate fixed time would indicate an apparent reduction in the extent of capping, as reported previously. Thus, alteration of the drug concentration and preincubation time can change the apparent combined effects of the drugs; such combination can therefore yield no useful information about the mechanism of capping.

Discussion

It is clear from the data presented that cap formation by different ligands shows a consistent pattern of responses to ligand concentration and drugs which affect the cytoskeleton, provided the kinetics of cap formation are taken into account. By appropriate adjustment of the concentrations of the ligand(s) cross-linking the receptors, capping of different receptors can be driven to similar maximal rates with half-times of 1.5–3 min. The effects of cytochalasin B and colchicine are generally on the rate, but not the final extent, of cap formation; when different receptors are capped at similar rates, then a given drug concentration generally has a similar effect on the capping rates.

Comparison with previous data

Most previous studies have measured the effects of cytochalasin B and colchicine at arbitrarily fixed times. Not surprisingly, the reported effects on the

extent of capping have varied widely, since they will depend on the ligand and drug concentrations used, and on the sampling time for the measurement of capping. Our data show that many of the substantial differences in the literature relating to cap formation by various ligands, and their sensitivity to cytochalasins and colchicine, can be rationalized by taking into account the different experimental conditions.

For example, the effects of cytochalasin B on the capping of sIg have been described as 'variable and not extensive' (Schreiner & Unanue, 1976) on the basis of work by several groups in which the apparent inhibition of cap formation was between 10 and 60% (De Petris, 1975; Edelman *et al.*, 1973; Schreiner & Unanue, 1976; Taylor *et al.*, 1971; Unanue *et al.*, 1974). In fact, by appropriate manipulation of the concentration of rabbit anti-mouse IgG and the sampling time, any result between no inhibition and complete inhibition can be generated for a given cytochalasin concentration (Fig. 5). Similar considerations apply to the reported effects of colchicine, previously observed to have little or no effect on sIg capping by rabbit anti-mouse IgG (Edelman *et al.*, 1973; Taylor *et al.*, 1971), but shown here to increase the rate of capping progressively with increasing concentration (Fig. 6). It is clear that this effect of colchicine would not be detected in experiments in which maximal cap formation had occurred in the control cells at the fixed sampling time (Edelman *et al.*, 1973; Taylor *et al.*, 1971). The combined effects of colchicine and cytochalasin B further demonstrate that apparently contradictory results can be obtained by altering the conditions (preincubation time, concentrations and sampling time).

The conflicting reports on whether rabbit anti-mouse IgG caps sIg on spleen cells much faster than it caps θ antigen pretreated with anti- θ antibody on thymocytes (Braun *et al.*, 1978a), or whether the capping rates are similar (Hoessli *et al.*, 1980), depend directly on the concentration of anti- θ antibody used with a given rabbit anti-mouse IgG concentration. Both results are valid, but any distinction between capping mechanisms on the basis of the rate of capping of the two receptors is not. Furthermore, the extent of θ antigen capping can be reduced to a relatively low level if a high ratio of anti- θ antibody to rabbit anti-mouse IgG is used [Fig. 3(b); compare Taylor *et al.* (1971)]. We assume that the rather complex relationship in this system between cap formation and the concentration of the primary and secondary ligands depends on the balance between intra- and inter-molecular crosslinking by the two antibodies of their respective determinants. We think that this phenomenon is similar in origin to the precipitation by cross-linking of antibody-antigen complexes:

precipitation occurs in solution at optimal proportions of the two reactants, but soluble complexes are formed at non-optimal ratios. A similar effect may account for the reduction in the extent of capping at high anti- θ antibody concentrations on T cells (Fig. 3b), and at very high concentrations of rabbit anti-mouse IgG on spleen B cells, as previously suggested by Taylor *et al.* (1971).

We suggest that the apparently conflicting reports for myosin redistribution and drug sensitivity of capping by different ligands (see Introduction) may also result from arbitrary differences in the experimental conditions used to measure capping. Thus, no myosin redistribution was reported for capping of θ at a slow rate (Braun *et al.*, 1978a), but α -actinin redistribution was observed when capping of θ occurred at a rate similar to that of sIg capping (Hoessli *et al.*, 1980). In our view, it is only valid to compare the sensitivity of different receptors to drugs under conditions where capping on control cells is occurring at a similar rate. In particular, we find that under such conditions A23187 (Pozzan *et al.*, 1980) and chlorpromazine (T. Pozzan & A. N. Corps, unpublished work) both block capping by all ligands by depletion of cellular ATP, and that there is therefore no evidence for different capping mechanisms on the basis of the action of these drugs on cap formation [as in Braun *et al.* (1978b)]. We can confirm that A23187 will disperse caps formed using a single ligand, but not two crosslinking layers. However, this may well reflect the extent of cross-linking in the caps, rather than any difference in the mechanisms by which they were formed.

Factors determining the rate of capping

In general, the overall rate of capping will depend on the rates of energy-independent patch formation and of the subsequent energy-dependent steps. The formation of patches on cells within 30 s of ligand addition under optimal conditions is compatible with free lateral diffusion of the receptors unhindered by 'spontaneous' attachment to the cytoskeleton (i.e. with diffusion rates $\approx 10^{-9} \text{ cm}^2 \cdot \text{s}^{-1}$). This is also consistent with evidence from photobleaching recovery experiments on sIg and θ antigen which indicate that most of the receptors (70–90%) undergo fast lateral diffusion at these rates (Dragsten *et al.*, 1979). The subsequent formation of caps from patches at similar maximum rates for the different receptors is compatible with a common mechanism for the energy-dependent step in capping.

In general, the rate of patch formation depends on the rate of cross-linking of the receptors, which will depend on the concentrations of the ligand and its receptors, and on their intrinsic mutual valencies. For example, we suppose that the reason why anti- θ antibody alone does not cause fast capping is that its

mutual valency with θ antigen is suboptimal. This is of interest in that the anti- θ antibody itself is a monoclonal IgM and therefore has five binding sites for its specific θ antigen determinant. If θ antigen was effectively monovalent, no patch of more than five receptors could be induced by the anti- θ antibody alone. We assume that either θ antigen is at least divalent (which would be sufficient to allow patch formation by the IgM antibody), or that it exists in an oligomeric form, or that two or more θ antigens may associate with other proteins to achieve effective polyvalency. The addition of the second antibody (rabbit anti-mouse IgG) with a sufficient effective valency for the anti- θ antibody, causes fast cross-linking at appropriate concentrations, so that this no longer the major rate determining factor, and capping then occurs at the maximal rate. We emphasize that, in this view, the only function of the second layer of antibody is to increase cross-linking efficiency to a level at which it is no longer the major rate determining factor: it does not alter the mechanism of capping.

Implications for models of capping

Two main types of models of cap formation have been proposed, both of which presume that the underlying mechanism is the same for all ligands. One set of models proposes that when surface receptors are crosslinked to form patches, they are recognized by cytoskeletal elements in the cell which attach to the patches and act in a concerted manner to draw them to a pole of the cell (Bourguignon & Singer, 1977; De Petris, 1975; Flanagan & Koch, 1978). The alternative models suggest that the process of capping reflects net movement of the cell membrane towards a pole, which carries with it cross-linked patches of receptors large enough to resist counter-diffusion by Brownian motion (Bretscher, 1976). Data in favour of the flow model based on the capping of exogenous Forssman glycolipid on cells has recently been put forward by Stern & Bretscher (1979). In the flow models, in which no specific recognition of cross-linking of the receptors by the cytoskeleton is required, the effects of drugs assumed to act on the cytoskeleton must be indirect; by affecting lipid or membrane flow they affect cap formation. In such models, the effects of a drug acting on the cytoskeleton would therefore be expected to be similar for cap formation by different receptors, provided that the capping occurred at a similar rate. The present study indicates a uniform effect of cytochalasin in slowing the rate of capping by three different receptors to similar extents. This is consistent with a common involvement of microfilaments (Mookerjee *et al.*, 1981; Wessels *et al.*, 1971) in driving capping and is compatible either with a role for the microfilaments in generating membrane flow, or with the attachment of the

cytoskeleton to patches before cap formation. On the other hand, the effect of colchicine is receptor-specific, in that it increases the rate of capping of sIg or concanavalin A receptors, but not of θ antigen, at any rate of cap formation. This is not readily compatible with simple flow models, and for models involving direct attachment of the cytoskeleton to the patches, requires that the precise nature of this attachment, or of the action of the cytoskeleton on the patches, depends on the nature of the receptors.

We do not regard this as a critical test of the models: the clearest distinction we can imagine would be to determine directly whether or not prior attachment of patches to the cytoskeleton is obligatory for capping.

We thank Dr. G. A. Smith for the preparation of labelled concanavalin A stocks. A. N. C. holds an MRC studentship and T. P. was the recipient of a long-term EMBO fellowship. The work was supported by a grant from the SRC to J. C. M., and the paper was written during tenure by J. C. M. of a John E. Fogarty Center Scholarship in Residence at the National Institutes of Health, from January to March, 1981.

References

- Ash, J. F., Louvard, D. & Singer, S. J. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5584–5588
- Bourguignon, L. Y. W. & Singer, S. J. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5031–5035
- Braun, J., Fujiwara, K., Pollard, T. D. & Unanue, E. R. (1978a) *J. Cell Biol.* **79**, 409–418
- Braun, J., Fujiwara, K., Pollard, T. D. & Unanue, E. R. (1978b) *J. Cell Biol.* **79**, 419–426
- Bretscher, M. S. (1976) *Nature (London)* **260**, 21–23
- De Petris, S. (1975) *J. Cell Biol.* **65**, 123–146
- Dragsten, P., Henkart, P., Blumenthal, R., Weinstein, J. & Schlessinger, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5163–5167
- Edelman, G. M., Yahara, I. & Wang, J. L. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1442–1446
- Flanagan, J. & Koch, G. L. E. (1978) *Nature (London)* **273**, 278–281
- Hesketh, T. R., Smith, G. A., Houslay, M. D., Warren, G. B. & Metcalfe, J. C. (1977) *Nature (London)* **267**, 490–494
- Hoessli, D., Rungger-Brandle, E., Jockusch, B. M. & Gabbiani, G. (1980) *J. Cell Biol.* **84**, 305–314
- Loor, F. (1974) *Eur. J. Immunol.* **4**, 210–220
- Loor, F. & Angman, L. (1980) *Exp. Cell Res.* **129**, 289–295
- Mookerjee, B. K., Cuppoletti, J., Rampel, A. L. & Jung, C. Y. (1981) *J. Biol. Chem.* **256**, 1290–1300
- Pozzan, T., Corps, A. N., Montecucco, C., Hesketh, T. R. & Metcalfe, J. C. (1980) *Biochim. Biophys. Acta* **602**, 558–566
- Pozzan, T., Corps, A. N., Hesketh, T. R. & Metcalfe, J. C. (1981) *Exp. Cell Res.* **134**, 399–408
- Rinderknecht, M. (1962) *Nature (London)* **193**, 167–168
- Schreiner, G. F. & Unanue, E. R. (1976) *J. Exp. Med.* **143**, 15–31
- Stern, P. L. & Bretscher, M. S. (1979) *J. Cell Biol.* **82**, 829–833
- Taylor, R. B., Duffus, W. P. H., Raff, M. C. & De Petris, S. (1971) *Nature (London) New Biol.* **233**, 225–229
- Unanue, E. R., Ault, K. A. & Karnovsky, M. J. (1974) *J. Exp. Med.* **139**, 295–312
- Wessels, N. K., Spooner, B. S., Ash, J. F., Bradley, M. O., Luduena, M. A., Taylor, E. L., Wrenn, J. T. & Yamada, K. M. (1971) *Science* **171**, 135–143