Role of a pertussis toxin substrate in the control of lectin-induced cap formation in human neutrophils

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We have examined the role of GTP-binding proteins and the associated cyclic AMP- and calcium-related transduction mechanisms in the regulation of capping in human neutrophils. Pertussis toxin (PT), a probe for the GTP-binding protein N_i , abolished capping induced by fluorescein isothiocyanate-conjugated concanavalin A (Con-A), whereas cholera toxin, a probe for the GTP-binding protein N_s , was without effect. Consistent with the latter finding, ligands acting at receptors associated with the N_s protein, namely the prostaglandin E_1 and β -adrenergic agonists, were without effect on the capping reaction. The possible role of mobilization of internal calcium was evaluated by using Quin2-loaded cells. Calcium mobilization was observed at concentrations of Con-A which yielded optimal capping ($10 \mu\text{g/ml}$). Treatment with PT, phorbol myristate acetate or 8-(*NN*-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) abolished both calcium mobilization and capping. Colchicine, which substantially enhanced capping, had no effect on calcium mobilization. At concentrations of the lectin above those required for capping, superoxide generation and enzyme release were noted. These reactions were less susceptible to inhibition by PT, effects being observed only on the K_{act} for Con-A-mediated superoxide generation with little effect on the V_{max} . The degree of PT-mediated inhibition for enzyme release with Con-A was much lower than that observed with fMet-Leu-Phe. Our results imply that (a) a step involving N_i -mediated calcium mobilization, sensitive to phorbol myristate acetate, is essential to the regulation of capping; (b) a distinct mechanism may be involved in colchicine-mediated enhancement of capping; and (c) N_i may play a relative minor role in the regulation of lectin-mediated exocytosis.

INTRODUCTION

Binding of lectins and immunoglobulins to cell surface binding sites results in a rearrangement of these ligand–receptor complexes to one end of the cell in a process known as capping (De Petris, 1975; Loor, 1974; Stackpole *et al.*, 1971; Taylor *et al.*, 1971). This reaction is important since it has served as a model for the study of the mobility of membrane proteins and also because several important functional responses can be initiated as a result of cell surface crosslinking. Recent results indicate that GTP-binding proteins are involved in the regulation of the human neutrophil (Koo *et al.*, 1983; Bokoch & Gilman, 1984; Lad *et al.*, 1985*b,c*; Okajima & Ui, 1984; Okajima *et al.*, 1985). A PT-sensitive GTP-binding protein is involved in the regulation of the FMLP receptor and in the control of diverse reactions controlled by the receptor including aggregation, enzyme release, superoxide generation and chemotaxis (Lad *et al.*, 1985*b*). It is also evident that several transduction systems are affected by PT. These include adenylate cyclase, phospholipase A_2 (Koo *et al.*, 1983; Lad *et al.*, 1985*a*; Okajima & Ui, 1984) (as judged by the release of arachidonic acid) and a polyphosphoinositide phosphodiesterase, which is important in the generation of inositol triphosphate (Cockcroft *et al.*, 1985; Vergheze *et al.*, 1985). In contrast to the involvement of the

PT-sensitive protein (termed variously N_i , N_o , N_p and N_x), the CT substrate in the neutrophil plasma membrane (termed N_s) regulates the PGE_1 and β -adrenergic receptors, which are involved in the elevation of cyclic AMP and in the inhibition of FMLP-mediated superoxide generation (Lad *et al.*, 1985*a*). These results prompted us to investigate whether the PT substrate and an associated calcium-mediated pathway may be involved in the regulation of lectin-induced capping in human neutrophils. Such an observation would be particularly important, as an inhibitor of the capping process would facilitate the analysis of molecular events involved in this complex reaction, and in the evaluation of some proposed hypotheses (Bretscher, 1984; Oliver *et al.*, 1982; Schreiner & Unanue, 1976; Bourignon & Bourignon, 1984). Our results point to the involvement of a PT substrate, associated with calcium mobilization, in the regulation of lectin-induced capping.

MATERIALS AND METHODS

Materials

BSA, PMA, cytochrome *c*, superoxide dismutase, cytochalasin B, Quin2, Quin2 AM, Hepes, colchicine, TMB-8, PGE_1 , isoproterenol and *Micrococcus lysodeikticus* were purchased from Sigma. Dextran T-500 and

Abbreviations used: FMLP, fMet-Leu-Phe (*N*-formyl-L-methionyl-L-leucyl-L-phenylalanine); BSA, bovine serum albumin; N_s and N_i , GTP-binding receptor regulatory proteins; HBSS, Hanks' balanced salt solution; CT, cholera toxin; PT, pertussis toxin; PAF, platelet activating factor; PMA, phorbol 12-myristate 13-acetate; Con-A, concanavalin A; FITC, fluorescein isothiocyanate; TMB-8, 8-(*NN*-diethylamino)octyl-3,4,5-trimethoxybenzoate; PGE_1 , prostaglandin E_1 ; RO20-1724, D-1,4-(3-butoxy-4-methoxybenzyl)-2-imidazolidine.

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Ficoll were obtained from Pharmacia, sodium diatrizoate (Hypaque) from Sterling and HBSS from Gibco. Con-A was purchased from E-Y Labs (San Mateo, CA, U.S.A.) and FITC-Con-A from Vector Labs (Burlingame, CA, U.S.A.). PT, purified by the method of Sekura *et al.* (1983), and CT were purchased from List Biologicals (Campbell, CA, U.S.A.). Additionally, the effects observed for PT have been highly specific for nucleotide regulatory proteins, and in experiments with plasma membranes, totally dependent on the presence of NAD⁺. RO20-1724 was generously given by Dr. Ann Welton, Hoffman-LaRoche, NJ, U.S.A.

Isolation of neutrophils

One-half volume of 4.5% dextran T-500 in 0.85% NaCl was added to heparinized (10 units/ml) whole blood (Boyum, 1968). The mixture was allowed to stand for 30 min at room temperature. The supernatant containing leucocytes was removed and layered on Ficoll/Hypaque (63.5 g of Ficoll and 100 g of Hypaque in 1 litre of water). The mixture was centrifuged at 500 *g* for 15 min at 4 °C. The pellet predominantly contained erythrocytes and neutrophils. The erythrocytes were lysed by the addition of 12 ml of water for 30 s, followed by the addition of 4 ml of 3.5% NaCl, mixed well, centrifuged at 250 *g* for 15 min and resuspended in modified HBSS (containing 1 mg of glucose/ml, 1 mg of BSA/ml and 10 mM-Hepes, pH 7.4). The cell preparation contained > 98% neutrophils. The functional properties of enzyme release, chemotaxis and superoxide generation were normal in neutrophils isolated by this procedure. The ranges for these responses at 10⁻⁶ M-FMLP were as follows: chemotaxis 300–400 migrating cells (> 20 µm); 32.2 nmol of O₂⁻/5 min per 10⁷ cells for superoxide generation; 20.4 µg equivalents of lysozyme/5 min per 10⁷ cells.

PT incubation

Whole cell treatments with PT were carried out by incubating the neutrophils (1 × 10⁸ cells/ml) with 15 µg of PT/ml at 37 °C, in modified HBSS containing 0.01 unit of heparin/ml for 30–120 min (Lad *et al.*, 1985b,c). The cells were then centrifuged at 250 *g* for 15 min and resuspended in modified HBSS without heparin and assayed. The control for the whole cell treatment consisted of treatment with buffer alone under identical incubation conditions and was included with every assay. Cell viability, checked routinely using Trypan Blue exclusion and assays for lactate dehydrogenase, was > 95% in both PT-treated and control cells. PMA-mediated generation of superoxide was used additionally as a rapid test for the functional integrity which was unimpaired in both control and toxin treated cells.

Neutrophil function tests

Enzyme release (Zurrier *et al.*, 1974). Neutrophils (4 × 10⁷ cells/ml) were diluted 2-fold into a solution containing 20 µg of cytochalasin B/ml in modified HBSS (Loor, 1974). Con-A (2–100 µg/ml) was added and the cells were incubated for a further 5 min at 37 °C. The cells were centrifuged at 400 *g* for 15 min at 4 °C and the supernatant containing the released enzymes was assayed for lysozyme. Total release was determined by incubation in a buffer containing 0.5% Triton X-100. The lysozyme

released by Con-A (20 µg/ml) was in the range 35–45% of the total.

Superoxide generation (Simchowitz & Spilberg, 1979). The reduction of cytochrome *c* was monitored for superoxide generation by using a fixed time point assay. Neutrophils (700 µl of 2 × 10⁷ cells/ml) were washed and resuspended in HBSS. Cytochalasin B (100 µl of 100 µg/ml) and cytochrome *c* (100 µl) were added to give a final concentration of 7.6 × 10⁻⁹ M (Mazurek *et al.*, 1984). The range of ligand concentrations was similar to that used in enzyme release. The cells were incubated at 37 °C for 5 min and then centrifuged at 3000 *g* for 30 s in a Beckman Microfuge. The supernatants were decanted and the amount of reduced cytochrome *c* read at 550 nm in a Gilford 2600 spectrophotometer. Controls included incubation of cells and substrate without ligand, and with ligand in the presence of superoxide dismutase. The reaction rate was linear over 5 min and values obtained in a continuous assay were within 10% of those obtained with the fixed time point assay. An absorbance change of 1.0 corresponds to the generation of 46 nmol by using the published absorption coefficient for cytochrome *c*.

FITC-Con-A-induced capping (Oliver *et al.* 1975, 1977). The FITC-Con-A used in this assay was active in superoxide generation and was more potent (up to 20-fold) than the native Con-A. Neutrophils were suspended in modified HBSS to a final concentration of 2 × 10⁷ cells/ml. The cells were preincubated with buffer, colchicine (10⁻⁵ M) or lumicolchicine (10⁻⁵ M) for up to 30 min at 37 °C. FITC-Con-A was then added at a concentration of 10 µg/ml and the capping reaction was initiated at 37 °C for the periods of time indicated in the individual experiments (up to 10 min). The cells were fixed with 2% paraformaldehyde for 10 min at 37 °C, and a wet mount was prepared using 10 µl of the cell mixture on a glass slide with a no. 1 cover slip sealed onto the slide with clear nail polish. The cells were then examined under oil, with a Zeiss epifluorescence microscope fitted with an FITC interference filter and a ×40 or ×60 objective. Scoring was done in two categories with respect to the distribution of the label as 'random clusters' (diffuse or patched) or 'capped'. Typically, the capped structure showed most of the label clustered to one end of the cell as a protuberance or bleb from the cell membrane. The random cluster is defined as a distribution of label in small aggregates throughout the surface of the cell, and also includes cells which have barely visible surfaces, with little local concentration of label. Incubation at 0 °C in the presence of FITC-Con-A was included as a control, and showed no capping. Lumicolchicine served as a satisfactory control for the effects of colchicine in the enhancement of cap formation. Enhancement of capping by colchicine was always observed, although the degree of enhancement was variable (70–100% capped cells). Similarly, inhibition of capping by PT was also observed in all experiments but the degree of inhibition ranged from 65 to 100%.

Measurement of intracellular calcium by Quin2 fluorescence

Fluorescence of Quin2-loaded cells was measured by the method of Tsien *et al.* (1982) and of Korchak *et al.* (1984). Neutrophils (10⁸ cells/ml) were loaded with Quin2

Fig. 1. Distribution of FITC-Con-A in human neutrophils

Control and PT-treated neutrophils were suspended in modified HBSS at a concentration of 5×10^6 cells/ml. The cells were then treated with buffer or colchicine (10^{-5} M) and subsequently exposed to FITC-Con-A ($10 \mu\text{g/ml}$). Panels indicate the following: control (a) and colchicine-treated (b) capped cells; PT-treated cells in the presence (c) and absence (d) of colchicine showing random clusters. The details of the PT treatment and the capping reaction are outlined in the Materials and methods section.

by incubating for 30 min at 37°C with Quin2 AM in Hepes buffer containing 150 mM-NaCl, 5 mM-KCl, 1.3 mM- CaCl_2 , 1.2 mM- MgCl_2 and 10 mM-Hepes, pH 7.4. The suspension was then diluted 10-fold and incubated for an additional 30 min at 37°C . After loading, the cells were centrifuged at $150 g$ for 10 min, resuspended in Hepes buffer at a concentration of 10^7 cells/ml, and kept at room temperature. The assay (37°C) was as follows: 1 ml of the cell suspension was diluted with 2.5 ml of the Hepes buffer. This mixture was transferred to a 1 cm cuvette and, after the reading stabilized, the instrument was autozeroed. The respective ligand ($35 \mu\text{l}$) was then added, and the fluorescence was followed from 100 to 500 s. Fluorescence intensities were measured in a Perkin-Elmer LS-5 fluorescence spectrophotometer using an excitation wavelength of 339 nm and an emission wave length of 492 nm. Zero suppression and an enhanced sensitivity setting (FS = 30) were utilized for optimal readings. The excitation slit was set to 5 nm and the emission slit to 20 nm to enhance sensitivity. A measure of the total Quin2 present in the cells was estimated by lysing the cells in the presence of 0.1% Triton X-100. No difference was noted in the amount of Quin2 loaded into PT-treated and control cells. The level of intracellular calcium was calculated from a standard curve of Quin2, KCl and different concentrations of calcium. The PT treatment was carried out by first incubating the cell suspension (1×10^8 cells/ml) with PT ($15 \mu\text{g/ml}$) for 30 min at 37°C , then by going directly into the Quin2 loading as described above.

RESULTS

The characteristics of lectin-induced capping in human neutrophils are shown in Fig. 1. Capping is associated with the concentration of the label (FITC-Con-A) at one end of the cell (panels a and b), and with increased spreading and shape change. In agreement with previous results (Oliver & Berlin, 1982; Schreiner & Unanue, 1976) capping was completely dependent on temperature, was enhanced by colchicine and the agent lumicolchicine was without effect. After treatment with PT, cap formation was substantially decreased and patched configurations (random cluster) are observed instead (panels c and d). PT-treated cells did not show the characteristic bleb formation or spreading that is observed in control cells.

The effect of PT, a specific probe for the GTP-binding protein N_i , was further evaluated as shown in Fig. 2. The sufficiency of the concentrations of CT and PT was assessed separately by the effects of these toxins on the regulatory proteins in isolated neutrophil plasma membranes. For CT this was assessed by the degree of ADP-ribosylation and the functional alterations in GTP activation of adenylate cyclase (Lad *et al.*, 1985b). The sufficiency of PT levels was assessed by the abolition of guanine nucleotide regulation of the receptor in the isolated membrane and the inhibition of the functional responses of FMLP receptor in whole cells (Lad *et al.*, 1985a). The time course of the toxin pretreatment shows that incubation periods of between 1 and 2 h are required

Fig. 2. Effect of PT pretreatment on FITC-Con-A-induced capping

(a) Neutrophils (5×10^6 cells/ml in modified HBSS) were treated with PT ($15 \mu\text{g/ml}$) for 0–120 min (■, △) and the capping reaction was observed at $10 \mu\text{g}$ of Con-A/ml in the presence (●, ■) and absence (△) of 10^{-5} M-colchicine (▲, control). (b) Cells were treated for 90 min with 0.1–15 μg of PT/ml and capping was measured in the presence (■) or absence (●) of colchicine (c). The time course of capping in control (▲, ■) and PT-treated (●, ○) cells ($15 \mu\text{g/ml}$, 90 min) in the absence (●, ○) and presence (▲, ■) of 10^{-5} M-colchicine is shown. Bars indicate the S.E.M. of triplicate measurements.

for PT to exert its effects (Fig. 2a). The kinetics are consistent with the observation made for the inhibition of chemotaxis and are in accord with the slow entry of the toxin into the cell. The dose-response of capping shows that at concentrations of PT of $< 1 \mu\text{g/ml}$, capping is inhibited both in the presence and absence of colchicine (Fig. 2b). The kinetics of capping show that the inhibition of capping is due to the reduction in the extent of cap formation and not due to the induction of unstable caps (Fig. 2c). In several such experiments complete abolition of capping was noted in the absence of colchicine and inhibition in the presence of colchicine was in the range 75–100%.

In sharp contrast with the effect of PT, CT was without effect (Table 1). The lack of involvement of the N_s component was further verified by testing for the functional effects on capping of the N_s -linked PGE_1 and β -adrenergic receptors, in the absence and presence of the phosphodiesterase inhibitor RO20-1724. Under condi-

Table 1. Influence of cholera toxin and activators of adenylate cyclase on FITC-Con-A-induced and cyclic AMP production

Neutrophils were treated sequentially with buffer or CT ($10 \mu\text{g/ml}$) for 90 min, then with buffer, isoproterenol (10^{-4} M), PGE_1 (10^{-5} M) or RO20-1724 ($10 \mu\text{g/ml}$) for a period of 10 min at 37°C . The cells were then exposed to FITC-Con-A ($10 \mu\text{g/ml}$) for 10 min and the percentage of cells capped ($\pm$ S.E.M. of triplicate measurements) was calculated, as described in the Materials and methods section. Cyclic AMP production was measured by the radioimmunoassay method.

Ligand	Cells capped (%)	Cyclic AMP production (pmol/5 min per 4×10^6 cells)
Buffer	27 ± 1	0.30
PGE_1	28 ± 2	0.75
Isoproterenol	24 ± 2	0.78
PGE_1 + RO20-1724	34 ± 3	5.50
Isoproterenol + RO20-1724	28 ± 1	0.92
RO20-1724	27 ± 1	0.45
CT	29 ± 1	0.60

tions which cause over 1000-fold increase in cyclic AMP levels (Lad *et al.*, 1985a), little or no effect on capping could be observed. Since a role for N_i in the mobilization of calcium is indicated by recent studies (Cockcroft *et al.*, 1985; Verghese *et al.*, 1985; Volpi *et al.*, 1985; Lad *et al.*, 1985b), intracellular calcium levels were assessed by using the fluorescent calcium chelator Quin2. A dose-dependent increase in Quin2 fluorescence was noted (Fig. 3a) at concentrations at which capping was observed. Pretreatment of cells with PT, which caused an abolition of lectin-induced capping, also inhibited lectin-induced alterations in Quin2 fluorescence (Fig. 3b).

Since the phorbol ester PMA has been recently demonstrated to inhibit calcium mobilization, the effects of this ligand were tested. Although a marked inhibition of both capping and calcium mobilization was noted (Fig. 4), the apparent K_i for capping ($0.5 \mu\text{g/ml}$) was lower than that noted for inhibition of calcium mobilization ($1.2 \mu\text{g/ml}$). Further evidence for the involvement of calcium mobilization in the capping process was obtained by using the agent TMB-8 (Fig. 4 inset). TMB-8, an agent which acts at an unknown site to inhibit calcium mobilization, also inhibited capping, the K_i ($50 \mu\text{M}$) being similar for both processes.

The agent colchicine enhances capping. A graded, non-hyperbolic dose-response curve was observed, possibly involving a progressive loss of mechanical restraint (Fig. 5). The question of whether this regulation of cap formation might involve calcium mobilization was also tested. Calcium mobilization tested in the presence of colchicine (in the absence of ligands) showed no effect. Tests were also carried out to see if additional calcium mobilization occurred after colchicine pretreatment in the presence of hormone (Con-A, PAF, or FMLP). Again, no enhancement was observed (results not shown). Our results suggest that colchicine-mediated enhancement of capping did not occur with measurable calcium mobilization. Furthermore, agents which elevate cyclic AMP, which were without effect on the capping

Fig. 3. Con-A concentration-dependence and inhibition by PT of calcium mobilization

In (a) neutrophils (3×10^6 cells/ml) in HEPES buffer were exposed to various concentrations of Con-A in the range 2–20 $\mu\text{g/ml}$ after loading with Quin2. Changes in Quin2 fluorescence were recorded on a Perkin-Elmer LS-5 fluorescence spectrophotometer as described in the Materials and methods section. A fluorescence change of 100 units corresponds to 255 nM free intracellular Ca^{2+} . The letters denote the following: A, 10^{-7} M-FMLP; B–E, Con-A at 20, 10, 5 and 2 $\mu\text{g/ml}$ respectively. In (b) control (●; buffer) and PT-treated (■) cells were loaded with Quin2, exposed to various concentrations of Con-A as indicated, and the fluorescence change was monitored. The fluorescence intensity at 200 s is represented. The experiment was repeated twice with similar results.

reaction in the absence of colchicine, produce some inhibition of the colchicine-mediated enhancement of the process (Fig. 5 inset). No inhibition of capping was noted with CT in the presence or absence of colchicine. The disparity in action of CT and other N_s -linked agents may derive in part from the slow action of the toxin, in contrast with the rapid rise in cyclic AMP observed with the other agents. Inhibition of capping was noted only in colchicine-treated cells, an effect probably related to the enhancing effect of colchicine rather than of the capping reaction itself.

Two phases of responses are noted with Con-A in human neutrophils. The first involves capping at low concentrations ($< 5 \mu\text{g/ml}$). The second, observed at higher Con-A concentrations ($> 5 \mu\text{g/ml}$), includes superoxide generation (Fig. 6a) and enzyme release (Fig. 6b). For Con-A-induced superoxide generation, the degree (25% inhibition) and pattern of inhibition observed with PT was different from that noted with

either FMLP (75% inhibition) or PAF (100% inhibition) (Fig. 6c). For the lectin, inhibition is predominantly a K_{act} effect with relatively little effect on the V_{max} . Thus complete inhibition of responses to PAF and FMLP can be observed under conditions of relatively little inhibition of the Con-A response. Differences between the chemoattractants (FMLP and PAF) and Con-A were also noted when enzyme release was examined: (a) inhibition of enzyme release was less marked than inhibition of superoxide generation for the same ligands, and (b) inhibition by PT of Con-A-mediated enzyme release (30% inhibition) was lower than observed with PAF or FMLP (65%).

DISCUSSION

The results presented suggest that a PT substrate, but not a CT substrate, is important to the regulation of capping. The identity of these substrates can be inferred from the previously reported ADP-ribosylation patterns (Lad *et al.*, 1984a,b, 1985c) observed in intact membranes, where [α -³²P]NAD⁺ labels a 42 kDa protein (N_s) in the presence of CT, and a 41 kDa protein in the presence of PT. Our results would therefore support a role for N_i but not for N_s in the regulation of capping. The lack of a role for N_s is further supported by the findings that N_s -linked receptors for PGE_1 and β -adrenergic agonists, which cause substantial elevation in cyclic AMP, do not modulate capping.

The question of the type of N_i -associated process that may be important to capping has also been examined. Recent studies have shown that the N_i protein is involved in the regulation of phosphatidylinositol 4,5-bisphosphate phosphodiesterase which results in the formation of inositol trisphosphate (Okajima *et al.*, 1985; Cockcroft *et al.*, 1985). In addition to PT, two other potent inhibitors of calcium mobilization, PMA and TMB-8, are also inhibitors of capping. Considered together, these results point to N_i -associated calcium mobilization as being an important event in the overall regulation of capping.

Two questions arise from these observations: (a) how do these diverse agents act to inhibit calcium mobilization and (b) how does calcium mobilization influence the capping reaction? The nature of the inhibition by PT, PMA and TMB-8 can be inferred in part from previous studies. PT has been shown to uncouple the FMLP receptor from its regulatory protein (Okajima *et al.*, 1985). The site of action of PMA is unknown, although activation of kinase C (Nishizuka, 1984) is very likely to be involved, either in the phosphorylation of N_i itself or in subsequent steps leading up to the formation of inositol trisphosphate (Berridge, 1984). TMB-8 has been shown to inhibit calcium mobilization, but its site of action remains unknown (Chiou *et al.*, 1975; Smith & Iden, 1980). Further understanding of the mechanisms by which these agents affect calcium mobilization should also provide insights into the steps involved in the capping reaction.

With regard to the role of calcium mobilization in regulating cap formation, two hypotheses emerge from recent work (Berridge, 1984). The first is that diacylglycerol derived from phosphoinositide hydrolysis and the released calcium act in concert to activate kinase C (Nishizuka, 1984) which in turn initiates cellular responses. The second hypothesis is that higher levels of

Fig. 4. PMA- and TMB-8-mediated inhibition of FITC-Con-A-induced capping and calcium mobilization

Human neutrophils (1×10^7 cells/ml) were treated with various concentrations of PMA for 5 min at 37°C , pelleted, and capping (■) was monitored for a period of 10 min as described in the Materials and methods section. A separate batch of similarly treated cells from the same donor was evaluated for changes in Quin2 fluorescence (●) as described in the Materials and methods section; 100% Quin2 fluorescence corresponds to a calcium concentration of 250 nM. Inset denotes TMB-8-mediated inhibition of Quin2 response (●) and capping (■). The results are representative of three experiments

Fig. 5. Regulation of colchicine-mediated enhancement of capping

Neutrophils (5×10^6 cells in modified HBSS) were treated with colchicine at the concentrations indicated. The number of capped cells (●) and alterations in Quin2 fluorescence (■) were examined as described in the Materials and methods section. The inset denotes the effect on capping, in the presence of 10 mM-colchicine, of various ligands [PGE_1 , isoproterenol (ISO), RO20-1724 (RO) and CT] which activate adenylate cyclase and elevate cyclic AMP levels. The ligand concentrations and cyclic AMP levels were the same as in Table 1. Bars denote S.E.M. of triplicate measurements.

Fig. 6. Con-A-mediated superoxide generation and enzyme release

Control (●) and PT-treated (■) cells were exposed to different concentrations of Con-A and the generation of superoxide (a) and release of lysozyme (b) were monitored. The cell concentration in each assay was 1×10^7 cells/ml and the details are described in the Materials and methods section. The experiment was repeated with cells from two different donors, and both measurements (enzyme release and superoxide generation) were measured simultaneously. A comparison of the PT-mediated inhibition of superoxide generation and enzyme release mediated by Con-A relative to FMLP and PAF is shown in (c). The maximal values for superoxide generation were: FMLP, 32.2; PAF, 30.1; and Con-A 32.5 nmol of $O_2^{\cdot -}$ /5 min per 10^7 cells and for enzyme release; FMLP, 30.4; PAF, 18.5; and Con-A 15.3 μ g of lysozyme equivalent/min per 10^7 cells.

intracellular calcium act directly to activate a calcium-sensitive enzyme. Our preliminary results suggest that the activation of kinase C may serve to inhibit rather than initiate complex cellular processes. For example, PMA, a known activator of kinase C in the neutrophil, is a

potent inhibitor of calcium mobilization and capping. Thus the direct activation of calcium-sensitive enzymes needs to be considered. Such an enzyme could be $Ca^{2+} + Mg^{2+}$ -ATPase, phosphodiesterase, adenylate and guanylate cyclases, or myosin light chain kinase, all of which are known to exist in calmodulin-sensitive forms.

The final target of either mechanism (calmodulin- or kinase C-mediated activation) is likely to be altered membrane-cytoskeleton interactions (Nelson *et al.*, 1983; Koch & Smith, 1978; Flanagan & Koch, 1978). PT, which inhibits capping, also inhibits shape change (Lad *et al.*, 1985b) and actin polymerization. These observations are significant as they point to a role for a receptor-associated GTP-binding protein as a key participant in membrane-cytoskeleton interactions.

Our results also suggest that components other than calcium mobilization may contribute to regulate capping (Montecucco *et al.*, 1980; Pozzan *et al.*, 1982; Mikkelsen & Schmidt-Ulrich, 1980; Klausner *et al.*, 1974). Colchicine-mediated enhancement in capping is not associated with a similar enhancement in calcium mobilization. PGE_1 and other agents which enhance cyclic AMP and which do not affect cap formation in the absence of colchicine, inhibit the effects of colchicine. While PT, PMA and TMB-8 inhibit capping, even in the presence of colchicine, it is interesting to note that the effects of colchicine itself do not involve calcium mobilization. A 'mechanical restraint' of cap formation has been hypothesized to involve microtubules (Schreiner & Unanue, 1976). The relief from this type of restraint might be independent of N_i .

Our results point to two distinct ranges in the actions of the lectin Con-A on neutrophil functions. With regard to the reactions of superoxide generation and enzyme release, we find that calcium mobilization is involved; however, the pattern of inhibition by PT is also different. These results imply that different mechanisms (or different combinations of similar mechanisms) may be involved in lectin-induced capping and exocytosis. Our preliminary results suggest that a calcium channel may contribute to the actions of receptors such as Con-A and heat-aggregated IgG. Thus inhibitors of calcium channels (verapamil and diltiazem) as well as blockers of potassium channels (triethylammonium) are potent inhibitors of capping. Interestingly, verapamil does not inhibit the action of FMLP in superoxide generation. Thus a role for membrane elements other than N_i seems to be indicated in the action of lectin receptors. In this overall context, susceptibility of inhibition to PT may serve in the future to distinguish between such elements and to assess the relative contribution of N_i in the overall regulation of any complex cellular process.

We are grateful to Jimmie Jefferson for secretarial assistance. This work was supported by National Institutes of Health grants AM30878, AM34550 and a grant from the American Heart Association (no. 757) and funds from a Biomedical Support grant RR05521-19 from the Kaiser Foundation Research Institute to P.M.L. This is publication no. 80 of the Kaiser Regional Research Laboratory.

REFERENCES

- Berridge, M. J. (1984) *Biochem. J.* **220**, 345-360
- Bokoch, G. M. & Gilman, A. G. (1984) *Cell* **39**, 301-308
- Bourguignon, L. Y. W. & Bourguignon, G. J. (1984) *Int. Rev. Cytol.* **87**, 195-224

- Boyum, A. (1968) *Scand. J. Clin. Lab. Invest. Suppl.* **97**, 77–89
- Bretscher, M. (1984) *Science* **224**, 681–686
- Chiou, C. Y. & Malagodi, M. H. (1975) *Br J. Pharmacol.* **53**, 279–285
- Cockroft, S., Barrowman, M. M. & Gomperts, B. D. (1985) *FEBS Lett.* **181**, 259–263
- DePetris, S. (1975) *J. Cell Biol.* **65**, 123–146
- Flanagan, J. & Koch, G. L. E. (1978) *Nature (London)* **273**, 274–278
- Klausner, R. D., Bhalla, D. K., Dragsten, P., Hoovers, R. L. & Karnovsky, M. J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 437–441
- Koch, G. L. E. & Smith, M. J. (1978) *Nature (London)* **273**, 274–278
- Koo, C., Lefkowitz, R. J. & Snyderman, R. (1983) *J. Clin. Invest.* **72**, 748–753
- Korchak, H. M., Rutherford, L. E. & Weissman, G. (1984) *J. Biol. Chem.* **259**, 4070–4075
- Lad, P. M., Glovsky, M. M., Smiley, P. A., Klempner, M., Reisinger, D. M. & Richards, J. H. (1984a) *J. Immunol.* **132**, 1466–1471
- Lad, P. M., Glovsky, M. M., Richards, J. H., Learn, D. B., Reisinger, D. M. & Smiley, P. A. (1984b) *Mol. Immunol.* **21**, 627–639
- Lad, P. M., Goldberg, B. J., Smiley, P. A. & Olson, C. V. (1985a) *Biochim. Biophys. Acta* **846**, 286–295
- Lad, P. M., Olson, C. V., Grewal, I. S. & Scott, S. J. (1985b) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8643–8647
- Lad, P. M., Olson, C. V. & Smiley, P. A. (1985c) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 869–873
- Loor, F. L. (1974) *Eur. J. Immunol.* **4**, 210–220
- Mazurek, N., Schindler, H., Schurholtz, T. & Pecht, I. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6841–6845
- Mikkelsen, R. B. & Schmidt-Ulrich, R. (1980) *J. Biol. Chem.* **255**, 5177–5183
- Montecucco, C., Rink, T. J., Pozzan, T. & Metcalfe, J. C. (1980) *Biochim. Biophys. Acta* **595**, 65–70
- Nelson, W. J., Colaco, C. & Lazarides, E. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1626–1630
- Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698
- Okajima, F. & Ui, M. (1984) *J. Biol. Chem.* **259**, 13863–13871
- Okajima, F., Katada, T. & Ui, M. (1985) *J. Biol. Chem.* **260**, 7226–7233
- Oliver, J. M. & Berlin, R. D. (1982) *Int. Rev. Cytol.* **74**, 55–94
- Oliver, J. M., Zurier, R. B. & Berlin, R. D. (1975) *Nature (London)* **253**, 471–473
- Oliver, J. M., Lalchandani, R. & Becker, E. (1977) *J. Reticuloendothel. Soc.* **21**, 357–364
- Pozzan, T., Arslan, P., Tsien, R. Y. & Rink, T. J. (1982) *J. Cell Biol.* **94**, 335–340
- Schreiner, G. F. & Unanue, E. R. (1976) *Adv. Immunol.* **24**, 37–165
- Sekura, R. D., Fish, F., Manclark, C. R., Meade, B. & Zhang, Y. L. (1983) *J. Biol. Chem.* **258**, 14647–14651
- Simchowitz, L. & Spilberg, J. (1979) *J. Immunol.* **123**, 2428–2435
- Smith, R. J. & Iden, S. S. (1980) *Inflammation* **4**, 343–358
- Stackpole, C. W., Aoki, T., Boyse, E. A., Old, L. J., Lumley, F. J. & DeHaaven, E. (1971) *Science* **172**, 472–474
- Taylor, R. B., Duffus, W., Raff, M. & DePetris, S. (1971) *Nature (London) New Biol.* **233**, 225–229
- Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982) *J. Cell Biol.* **94**, 325–334
- Verghese, M. W., Smith, C. D. & Snyderman, R. (1985) *Biochem. Biophys. Res. Commun.* **127**, 450–457
- Volpi, M., Naccache, P. H., Molski, T. F. P., Shefcyk, J., Nuang, C.-K., Marsh, M. L., Munoz, J., Becker, E. L. & Sha'afi, R. I. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2708–2712
- Zurier, R. B., Weissman, G., Hoffstein, S., Kammerman, S. & Tai, H. (1974) *J. Clin. Invest.* **43**, 297–309

Received 13 January 1986/7 March 1986; accepted 9 April 1986