A step sensitive to pertussis toxin and phorbol ester in human neutrophils regulates chemotaxis and capping but not phagocytosis

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Received 11 March 1986

Treatment of human neutrophils with pertussis toxin (PT) abolishes chemotaxis in response to either platelet-activating factor (PAF) or f-Met-Leu-Phe (FMLP), and capping induced via the concanavalin A (Con A) receptor. These functional effects are accompanied by the inhibition of calcium mobilization by PAF, FMLP and Con A. The agent phorbol 12-myristate-13-acetate (PMA) also inhibits chemotaxis and capping as well as calcium mobilization by these receptors. In sharp contrast, neither PT, cholera toxin (CT), nor PMA, inhibits the phagocytosis of non-opsonized and opsonized *Candida albicans*, sheep erythrocytes or fluorescent latex beads. Our results suggest that receptor-initiated chemotaxis and capping involve a step that is sensitive to PT and PMA, and that phagocytosis is not regulated in a similar fashion.

Ca^{2+} mobilization (Human) Neutrophil GTP-binding protein

1. INTRODUCTION

It is now evident from the work carried out in several laboratories [1–9] that a pertussis toxin (PT) substrate in the human neutrophil regulates multiple receptors and functions. Overall, the process probably involves a mechanism of phosphoinositide hydrolysis, the generation of inositol triphosphate (IP_3), and calcium mobilization [1–13]. The importance of the PT substrate in the regulation of multiple receptors raises the question of which functions are coordinated by this substrate and which other functions might be excluded from its control. This question is particularly important as several hypotheses have been proposed which connect chemotaxis, capping and endocytosis [14–16], although the molecular basis for the linkage of selective cell functions has remained unknown. Here, we have utilized PT to probe the range of involvement of its substrate in the regulation of chemotaxis, capping and phagocytosis. Our results suggest that a PT-substrate-associated calcium mobilization step is important to the regulation of capping and chemotaxis but is less significant in the regulation of phagocytosis.

2. MATERIALS AND METHODS

2.1. *Materials*

Fluorescein isothiocyanate (FITC) latex beads were purchased from Polysciences (Warrington, PA), and sheep erythrocytes from Colorado Serum (Denver, CO). The anti-sheep erythrocyte IgG was from Cordis (Miami, FL). *Candida albicans* was taken from Kaiser Hospital reference cultures. The purchase of all other materials has been described [7].

2.2. *Isolation of neutrophils*

Neutrophils were isolated by sequential centrifugation on dextran and Ficoll/Hypaque and toxin treatment of the cells was as described [7,17]. Toxin-treated cells were judged viable by exclusion of trypan blue and by phorbol 12-myristate

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00145793/86/$3.50

13-acetate (PMA) mediated superoxide generation, which was unaffected by the toxin.

2.3. *Chemotaxis*

The chemotaxis assay was performed in triplicate as follows [18]: 250 μl buffer (control well for random migration), or FMLP or PAF (1 nM–1 μM) were added to the bottom wells of the modified Boyden Chambers. Millipore filters (13 mm diameter, 3 μm pore size) were placed on top of the wells containing ligands, the upper chamber affixed, and 250 μl of the cell suspension (5×10^6) cells per ml in modified Hank's balanced salt solution (HBSS) added to the top wells. The chamber was placed in a moist 5% CO_2/95% air incubator at 37°C for 60 min. The filters were removed, stained and the slides counted as in [7].

2.4. *FITC-Con A capping*

Neutrophils (2×10^7 cells per ml in modified HBSS) were exposed to FITC-Con A (10 μg/ml). The reaction was terminated by the addition of 2% (w/v) paraformaldehyde. Wet mounts were examined for capping as described [7,19].

2.5. *Measurement of intracellular calcium using quin2 fluorescence*

Fluorescence of quin2-loaded cells was measured by the method of Tsien et al. [20,21]. Neutrophils (10^8 cells/ml) were loaded with quin2 by incubation for 30 min at 37°C with quin2 acetomethoxy ester 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. After washing, the ligand was added and the fluorescence followed from 100 to 500 s as in [7,10,21].

2.6. *Phagocytosis*

Systems utilized include uptake of sheep erythrocyte-antibody complexes (EA), FITC-conjugated *C. albicans* and FITC-conjugated latex beads.

2.7. *Assay for EA uptake by neutrophils*

Opsonized sheep erythrocytes were prepared as described [22–24]. Control or toxin-treated neutrophils (2 ml, 1×10^7 cells/ml in modified HBSS) were layered on clean glass cover slips (22 × 22 mm) arranged at the bottom of a 35 mm culture dish and incubated for 30 min at 37°C in a humidified 5% CO_2/95% air incubator to form monolayers on the cover slips. The monolayers were washed twice with modified HBSS and layered with 2 ml EAs (2×10^8 cells/ml in modified HBSS) and further incubated for the indicated time. The cover slips were removed, washed with PBS, dipped for 10 s into distilled water to lyse the attached erythrocytes, and fixed with 2% paraformaldehyde for 10 min at room temperature. The cover slips were then washed, mounted, and examined promptly using a phase-contrast Zeiss microscope and an oil immersion lens (63 ×). Cells were scored with ingested erythrocytes which appeared dark brown.

2.8. *Phagocytosis of* C. albicans

An FITC-labeled preparation of *C. albicans* was prepared as in [25,26]. Control or toxin-treated neutrophils (0.9 ml, 1×10^7 cells/ml) were mixed with 0.9 ml micro-organisms (1×10^8 organisms/ml) and 0.2 ml heat-treated human serum. All reactions were carried out in duplicate and a blank with no neutrophils was incorporated as well as one with no micro-organisms. The tubes were incubated for the times indicated at 37°C with continuous slow mixing. At the times indicated, 0.1 ml aliquots were removed and fixed with 2% paraformaldehyde for 10 min at 37°C. Wet mounts were prepared and slides examined with an epifluorescence Zeiss microscope using an oil immersion lens (63 ×).

2.9. *Phagocytosis of polystyrene particles*

Control and toxin-treated neutrophils (1 ml, 1×10^6 cells/ml) were incubated with 1 ml FITC-labeled polystyrene latex beads (5×10^7 spheres/ml) at 37°C for the times indicated with continuous slow mixing [27]. All assays were done in duplicate. The cells were then fixed with 2% paraformaldehyde for 10 min at 37°C.

2.10. *Calculation of phagocytosis data*

Data were expressed to determine the percentage of cells ingesting particles and the average number of particles ingested per cell. The phagocytic index is the number of particles ingested per 100 neutrophils. Both measures are utilized in the described experiments.

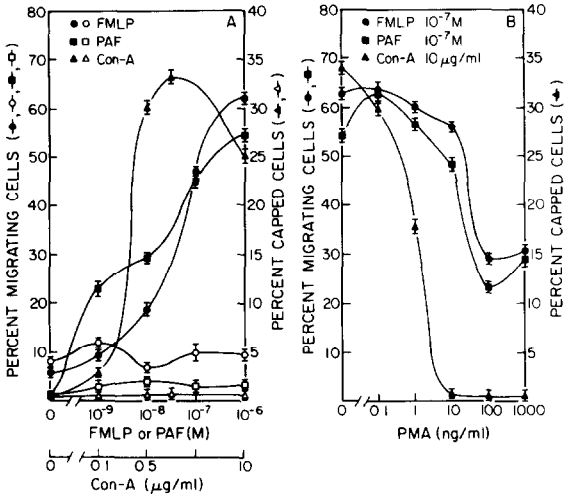

Fig.1. Effects of PT and PMA on chemotaxis and capping. Neutrophils were preincubated with PT or PMA, with a corresponding buffer incubation for control cells, and subsequently examined for chemotaxis and capping. The ligands tested in the chemotactic assay were FMLP and PAF, while capping was examined with FITC-Con A. The percentage of migrating cells was defined as those cells (percentage of total) moving a distance of 20 μm or greater in the Boyden chamber assay. The percentage of capped cells is defined as that fraction of total cells which had clear protuberance development with attendant shape change. Experiments were carried out to examine chemotactic and capping responses at different concentrations of the ligand in control (closed symbols) and PT-treated cells (open symbols, 15 μg/ml for 90 min at 37°C) (A); and in PMA-pretreated cells (5 min at 37°C) (B). Details of the chemotaxis and capping assays and treatment conditions for PT and PMA pretreatments are described in section 2.

3. RESULTS

Human neutrophils were pretreated with buffer or PT and the concentration dependence of PAF- and FMLP-mediated chemotaxis and Con A-induced capping examined (fig.1A). Under similar conditions, both chemotaxis and the ability of the lectin receptor to form caps was reduced in PT-treated cells. Similar experiments were carried out with cells exposed to differing concentrations of PMA (fig.1B). A dose-dependent inhibition of both responses was noted in PMA-treated cells.

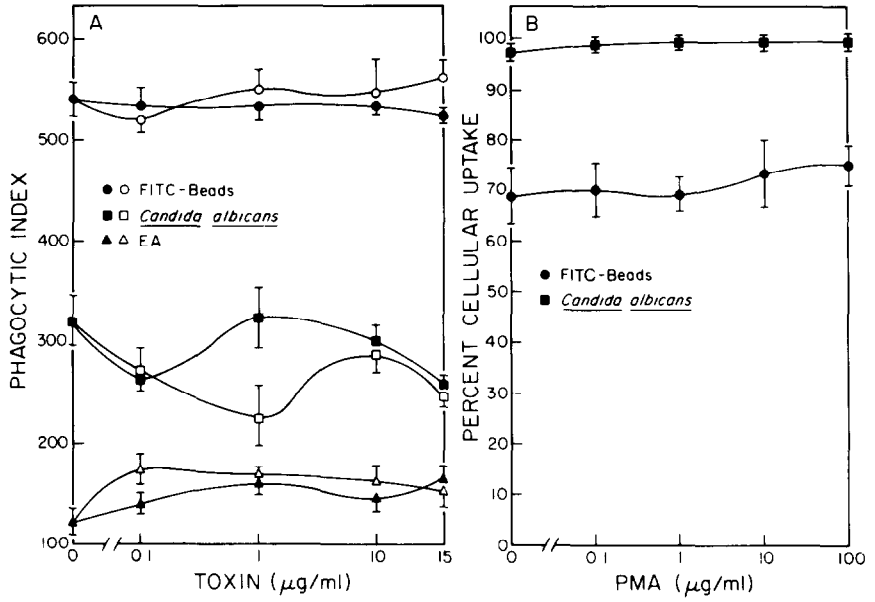

Fig.2. Effects of PT and PMA on phagocytosis. Control, CT-, PT- and PMA-pretreated neutrophils were incubated with either FITC-conjugated latex beads, opsonized FITC-conjugated *C. albicans*, or opsonized antibody-coated erythrocytes. The phagocytic index of CT- (closed symbols), or PT-treated (open symbols) cells (A), or the percent cellular uptake (B) was monitored as described in section 2.

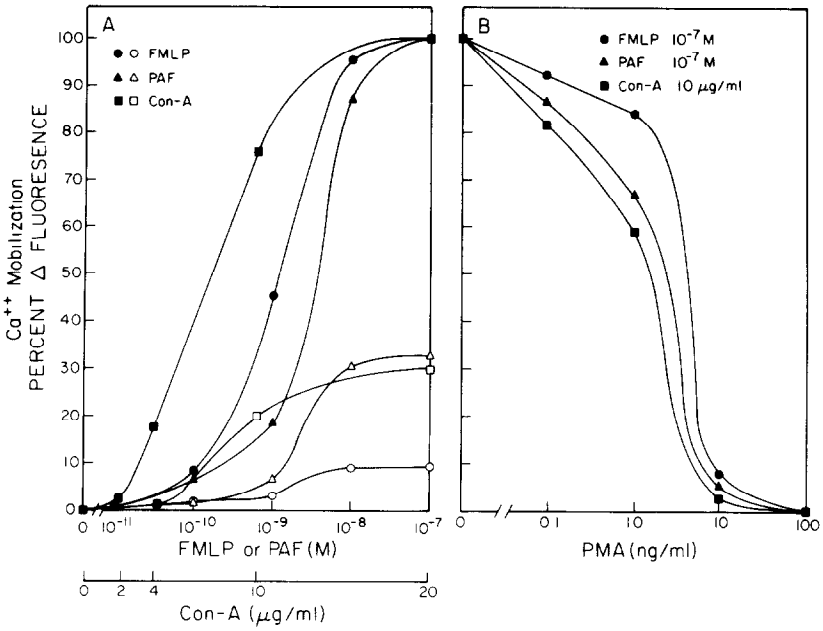

Fig.3. Effect of PT and PMA on calcium mobilization. Human neutrophils, loaded with the fluorescent calcium chelator quin2, were pretreated with buffer (closed symbols), PT (open symbols) (A), or PMA (B), under the conditions described in section 2. The change in fluorescence intensity in response to the different ligands was monitored continuously and the value at the 200 s (FMLP or PAF) or 400 s (Con A) mark is presented in the above figures. The details of the quin2 measurements are given in section 2. The concentration of intracellular calcium corresponding to the 100% mark was 250 nM (FMLP), 225 nM (PAF) and 280 nM (Con A).

Phagocytosis was then studied with FITC-conjugated latex beads and *C. albicans* as well as opsonized (antibody-coated) sheep erythrocytes (fig.2). In sharp contrast to the results obtained with chemotaxis and capping, neither PT or CT (panel A), nor PMA (panel B) produced inhibition of phagocytosis at those concentrations which completely suppress the former responses.

Mobilization of calcium via the receptors for PAF, FMLP and Con A was then examined using quin2-associated fluorescence (fig.3). Inhibition of calcium mobilization was noted for all 3 receptors in response to treatment with PT (panel A) as well as with PMA (panel B).

4. DISCUSSION

Our results indicate that a PT substrate is involved in the regulation of chemotaxis and capping. Since pertussis toxin also inhibits calcium mobilization from the receptors which initiate these processes, a PT substrate related calcium mobilization step would seem to be shared by these two processes.

The inhibition by PMA provides further evidence in favor of this hypothesis. The results of Sha'afi et al. [10,28] have shown that PMA is a potent inhibitor of calcium mobilization from the FMLP receptor. A simple prediction from this result would be that other receptors which mobilize calcium may also be similarly affected. Our results clearly suggest that this is a general phenomenon which applies to the PAF and Con A receptors. An important correlate of this observation should be that functional responses associated with these receptors should be inhibited. This is observed for PAF-mediated chemotaxis and Con A-induced capping. The possibility that kinase C may modify the CT- and PT-sensitive GTP-binding receptor regulatory proteins (N_s/N_i components, respectively) [29] is certainly consistent with our results.

Phagocytosis is unaffected in neutrophils in which the capping and chemotactic responses are inhibited by PT and PMA pretreatment. This is

observed regardless of the type (latex beads, *Candida*, erythrocytes) and state (opsonized or non-opsonized) of the particles presented to the cells. This result is important as it suggests that this process is not under the control of the PT (or CT) substrate and thus offers a clear limit to the involvement of this substrate on cellular functions.

Recent studies have indicated that cell spreading, as well as phagocytosis, involves low levels of calcium mobilization although a phagocytic mechanism independent of calcium mobilization has also been observed [30]. Our results suggest that either the pathway which is independent of calcium mobilization may be predominant, or calcium mobilization induced in response to surface or particle contact may not be under the control of the PT substrate. Measurement of calcium mobilization by monitoring the changes in fluorescent dyes in single cells [30] will be required to test which of these hypotheses may be applicable.

The result presented here allows for an evaluation of hypotheses offered in the literature [14–16] that certain processes involving the plasma membrane may be linked to each other through shared structural elements either in the plasma membrane or in the cytoskeleton. The nature of such a linkage has, however, remained obscure. Our results imply that chemotaxis and capping act through a PT substrate, possibly involving the control of cytoskeletal organization. Consistent with this possibility we find that shape change is under the control of the PT substrate [7]. Additionally, actin polymerization in response to cell surface receptors is also suppressed by the toxin [7,8]. Thus, a step from the PT substrate to actin polymerization, probably through a mechanism in which mobilized calcium plays an important role, may serve to integrate chemotaxis and capping. That phagocytosis is unaffected implies that receptors associated with this process (a subset of Fc gamma, mannose 6-phosphate, or C3b) might not be associated with the types of reactions observed with the PT substrate-mediated regulator of the lectin, PAF and FMLP receptors.

A more cautious interpretation of the role of 'kinase C' in neutrophil functions is essential. Phorbol esters clearly activate kinase C in neutrophils and in other cells [31]. However, such an activation would seem to inhibit rather than potentiate cellular responses such as chemotaxis, capping and associated calcium mobilization. These results are difficult to reconcile with the currently favored mechanism for chemoattractant receptors in which calcium mobilization together with diacylglycerol produced from hydrolyzed phosphoinositides serves to activate a putative kinase C and initiate cellular responses. Two possibilities should be considered: (i) that distinct kinase C enzymes initiate and regulate neutrophil functions; or (ii) that calcium mobilization mediated by chemotaxis and capping linked receptors may result in an enzyme activation other than that of kinase C, perhaps by acting directly on calcium (e.g. calmodulin) sensitive enzymes.

ACKNOWLEDGEMENTS

We are grateful to Jimmie Jefferson for secretarial assistance. This work was supported by grants to P.M.L. from the National Institutes of Health grants AM30878, AM34550; from the American Heart Association (no.757) and from a Biomedical support grant (RR05521-19) from the Kaiser Foundation Research Institute. This is publication no.91 of the Kaiser Regional Research Laboratory.

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