Treatment of rabbit neutrophils with phorbol esters results in increased ADP-ribosylation catalyzed by pertussis toxin and inhibition of the GTPase stimulated by fMet-Leu-Phe

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Received 12 December 1985; revised version received 29 January 1986

The effects of pretreatment of rabbit neutrophils with phorbol 12-myristate 13-acetate on the ability of pertussis toxin to catalyze ADP-ribosylation and of fMet-Leu-Phe to activate a high-affinity GTPase in these cell homogenates were examined. The addition of phorbol 12-myristate 13-acetate, but not 4α-phorbol 12,13-didecanoate, to intact cells was found to stimulate by more than 100% the pertussis toxin-dependent ribosylation of a 41 kDa protein (either the α-subunit of the 'inhibitory' guanine nucleotide-binding protein N_i or a closely analogous protein) and to inhibit by more than 60% the activation by fMet-Leu-Phe of the GTPase of the neutrophil homogenates. The addition of fMet-Leu-Phe to intact cells increases the ADP-ribosylation catalyzed by pertussis toxin of the 41 kDa protein. On the other hand, the exposure of neutrophil homogenates to fMet-Leu-Phe results in a decreased level of ADP-ribosylation. This decreased ribosylation reflects a dissociation of the GTP-binding protein oligomer that is not followed by association, possibly because of the release of the α-subunit into the suspending media. The implications of these results for the understanding of the mechanism of inhibition of cell responsiveness by phorbol esters and the heterologous desensitization phenomenon are discussed. Prominent among these are the possibilities that (i) the rate of dissociation of the N_i oligomer is affected by the degree of its phosphorylation by protein kinase C, and/or (ii) the dissociated phosphorylated α-subunit (the 41 kDa protein) is functionally less active than its dephosphorylated couterpart.

Phorbol ester *Pertussis toxin* *ADP-ribosylation*

1. INTRODUCTION

Much of the current interest in the field of signal transmission in calcium mobilizing systems is focused on the roles of guanine nucleotide-binding regulatory proteins in the mediation of the receptor-dependent stimulated hydrolysis of the inositol lipids [1]. The evidence linking a 'G protein' in such a process includes the demonstration of the potentiating effects of guanine nucleotides on the activity of phospholipase C in isolated membrane preparations [2–4] and the inhibitory activity of pertussis toxin towards the stimulation of inositol lipid breakdown and cell responsiveness by various agonists (e.g. in the neutrophils [5–11]).

It has been observed in several systems that the activation of protein kinase C by phorbol esters led to a decreased stimulation of the phospholipase C specific for the polyphosphoinositides and thus to impaired signal transmission and cell responsiveness [12–17]. The wide occurrence of this phenomenon has raised the possibility that one of the major physiological functions of the stimulation of protein kinase C is to generate negative, or termination, signal(s).

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

In this study we have investigated the possible effect of protein kinase C on the physical and functional state of the substrate of pertussis toxin in rabbit neutrophils. The approach used was to preincubate the cells with phorbol 12-myristate 13-acetate (PMA) and then to examine the ability of (i) pertussis toxin to catalyze the ADP-ribosylation of the α-subunit of N_i and (ii) fMet-Leu-Phe to stimulate the activity of the membrane-bound high-affinity GTPase in cell homogenates. The results to be described demonstrate that (i) preincubation of the cells with PMA or fMet-Leu-Phe before homogenization results in increased pertussis toxin-catalyzed ADP-ribosylation and inhibition of the stimulation by fMet-Leu-Phe of the high-affinity GTPase of these cell homogenates and (ii) addition of fMet-Leu-Phe to cell homogenates decreased the level of ADP-ribosylation catalyzed by pertussis toxin.

2. MATERIALS AND METHODS

Rabbit peritoneal neutrophils elicited 4 or 16 h previously by the injection of sterile isotonic glycogen solution were used throughout these experiments. They were collected, washed and resuspended in Hanks' balanced salt solution buffered with 10 mM Hepes as described [18].

The method for measuring pertussis toxin-catalyzed ADP-ribosylation was adapted from those published by Okajima and Ui [6] and Goldman et al. [11] with some modification. Cell suspensions (10^7 cells/ml) were treated with either 50 ng/ml PMA or the equivalent amount of 0.05% dimethylsulfoxide for 3 min. The cells were then washed once, resuspended in 0.1 M sucrose containing 5 mM Tris-HCl, pH 7.2, 2 mM EDTA and 0.5 mM DFP, and homogenized with a motor-driven teflon pestle. The homogenates were then centrifuged at 750 × g for 10 min and the supernatants used for ribosylation. The latter was started by the addition of 17 μg/ml of activated pertussis toxin (10 min incubation at 30°C with 10 mM dithiothreitol in 100 mM potassium phosphate buffer, pH 7.5) in a medium containing 0.1 M sucrose, 0.67 mM EDTA, 5 mM Tris-HCl, pH 7.2, 1 mM ATP, 2.5 mM $MgCl_2$, 10 mM thymidine, 0.5 mM DFP and 12 μM [^{32}P]NAD. The reactions were terminated by the addition of 25 μl of a stopping solution containing 9% SDS, 15% glycerol, 86 mM Tris-HCl, pH 6.7, 0.05% bromophenol blue, 6% β-mercaptoethanol to a 50 μl sample immediately followed by boiling for 20 min. The samples were then applied to straight 10.5% acrylamide gels. The gels were dried and the radioactivity visualized upon exposure of a Kodak XAR-5 film.

The activity of the GTPase was assayed as follows. Neutrophil homogenates were prepared by homogenization with a motor-driven teflon pestle from 10^8 cells (untreated, PMA or 4-α-PDD treated) resuspended in 1.0 ml of sucrose buffer (0.1 M sucrose, 2 mM EDTA, 10 mM Hepes) containing 0.5 mM DFP. The homogenates were then centrifuged at 750 × g for 10 min. Aliquots of the supernatants were diluted 20-times in Hanks' balanced salt solution containing 2.5 mM Mg^{2+}. The reaction mixture contained 0.52 ml homogenate supernatants diluted as just described, 0.2 ml substrate (2.0 mM App[NH]p, 2.0 mM $MgCl_2$, 4 mM dithiothreitol, 1.0 mM ATP, 20 mM phosphocreatine, 200 units/ml creatine phosphokinase, 1.0×10^{-6} M GTP and 1.0×10^{-6} M [^{32}P]GTP) and 0.08 ml of buffer or fMet-Leu-Phe. At the desired time, 0.1 ml aliquots were removed and added to tubes containing 1 ml of a charcoal solution (5% charcoal; 0.1% dextran, M_r 200000; 0.02% NaN_3; 0.5% bovine serum albumin and 20 mM potassium phosphate, pH 7.5). The tubes were then vortex-mixed, kept on ice for at least 5 min and centrifuged. Aliquots of the supernatants and of the total suspension mixture were then removed and counted. The GTPase assay described above represents a modification of that of Okajima et al. [19].

fMet-Leu-Phe was obtained from Peninsula Labs (San Carlos, CA), PMA from Calbiochem (San Diego, CA), 4-α-PDD from CMC Cancer Research Chemicals (Brewster, NY), ^{32}P-labelled GTP and NAD from New England Nuclear (Boston, MA). Pertussis toxin was a generous gift from Dr J.J. Munoz (NIH Rocky Mountain Lab., Hamilton, MT).

3. RESULTS AND DISCUSSION

In an effort to characterize the site of action of phorbol esters as inhibitors of stimulated neutrophil responsiveness, we have first examined the effect of preincubation with PMA on the

subsequent ability of pertussis toxin to catalyze ADP-ribosylation reactions in cell homogenates. The autoradiographic pattern of cell homogenates obtained from untreated, PMA treated or 4-α-PDD treated cells incubated with [^{32}P]NAD in the presence or absence of pertussis toxin is illustrated in fig.1. It is clear that pertussis toxin specifically catalyzes the ribosylation of a single protein band of relative molecular mass 41 kDa in rabbit neutrophil homogenates. Two-dimensional analysis revealed that this band had a p*I* of 5.7 (not shown). Bands of the same M_r (but of undetermined p*I*) have also been found to serve as substrates for pertussis toxin-catalyzed ADP-ribosylation in membranes from guinea pig and human neutrophils and have been identified tentatively as the α-component of the inhibitory guanine nucleotide-binding protein [6,7,11]. No ribosylation of the 41 kDa band can be detected in the absence of the toxin (whether or not PMA is present). Preincubation of the cells before homogenization with PMA can be seen to cause an increase in the autoradiographic density of the 41 kDa protein, an effect that was not observed when 4-α-PDD was used. It is worth noting that the incubation conditions with PMA used here are identical to those that had been characterized

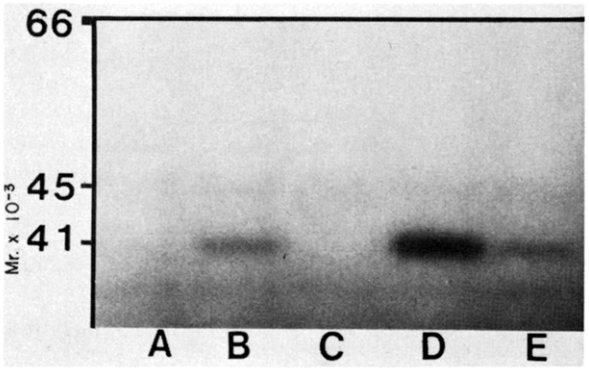

Fig.1. Effect of PMA and 4-α-PDD on the ADP-ribosylation catalyzed by pertussis toxin in rabbit neutrophil homogenates. The experimental conditions were as follows: A and B, control cells; A, no pertussis toxin; B, 17 μg/ml pertussis toxin; C and D, cells treated with 50 ng/ml PMA for 3 min before homogenization; C, no pertussis toxin; D, pertussis toxin; E, cells treated with 50 ng/ml 4-α-PDD before homogenization + pertussis toxin. Representative of at least 3 separate determinations.

Table 1

Effect of preincubation of the rabbit neutrophils with phorbol esters on the basal and pertussis toxin-induced ADP-ribosylation of the 41 kDa protein

Condition[a]	Amount of 41 kDa ribosylation (relative to control)	
	Basal	+ pertussis toxin[b]
Control cells	0	1.0 ± 0.10 (4)
+ PMA (50 ng/ml)	0	2.0 ± 0.30 (5)
+ 4-α-PDD (50 ng/ml)	0	0.94 ± 0.20 (3)

[a] The cells were preincubated with PMA or 4-α-PDD for 3 min before they were homogenized. No increase in ribosylation was observed when PMA was added after homogenization. The control cells have the same amount of DMSO (0.05%) as the phorbol ester treated cells

[b] The amount of pertussis toxin was 17 μg/ml and the reaction was carried out for 20 min. The values refer to mean ± SE, and the number in parentheses refers to the number of experiments. Each experiment was done in duplicate. No detectable ribosylation was observed in the absence of pertussis toxin

previously as optimal for inhibition of the stimulation of neutrophil responsiveness by chemotactic factors [16].

Table 1 summarizes in quantitative terms the effect illustrated in fig.1. The data presented here were derived from the densitometric scanning of autoradiograms similar to the one shown in fig.1. PMA can be seen to double the density of the 41 kDa protein while 4-α-PDD causes no significant change from the control ribosylation.

The effects of the chemoattractant fMet-Leu-Phe on the ADP-ribosylation of the 41 kDa protein were investigated next. The results summarized in table 2 show that the addition of fMet-Leu-Phe to intact neutrophils prior to homogenization results in increased ADP-ribosylation catalyzed by pertussis toxin, an effect similar to that described above with PMA. On the other hand, the addition of fMet-Leu-Phe to cell homogenates can be seen to induce a significant decrease in pertussis toxin-catalyzed ribosylation, a probable index of dissociation of the guanine nucleotide-binding protein oligomer.

Next we attempted to determine if the increased

Table 2

Effects of the addition of fMet-Leu-Phe to intact rabbit neutrophils or neutrophil homogenates on the ADP ribosylation of the 41 kDa protein catalyzed by pertussis toxin

Condition	Amount of 41 kDa ribosylation (relative to control)	
	Basal	+ pertussis toxin[a]
Control cells	0	1.0 ± 0.15 (3)
fMet-Leu-Phe added to intact cells[b]	0	1.45 ± 0.10 (2)
fMet-Leu-Phe added to cell homogenates[c]	0	0.48 ± 0.18 (3)

[a] The samples were treated with 17 μg/ml pertussis toxin for 20 min. The values refer to the mean ± SE and the number in parentheses refers to the number of experiments each carried out in duplicate

[b] The concentration of fMet-Leu-Phe was 10^{-7} M and the incubation time 1 min

[c] The concentration of fMet-Leu-Phe was 10^{-7} M, and it was present during the ribosylation reaction

ribosylation induced by PMA, that has just been described, resulted in altered functional properties of the putative G protein. The selected test of this hypothesis was to examine the effect of pretreatment with PMA on the stimulation of the high-affinity GTPase described by Hyslop et al. [20] and Okajima et al. [19]. The latter activity appears to be associated with N_i insofar as its stimulation by chemoattractants such as fMet-Leu-Phe is inhibited by pertussis toxin and increased upon addition of purified platelet N_i to the neutrophil membranes.

The results summarized in table 3 show that fMet-Leu-Phe increases the GTPase activity of rabbit neutrophil homogenates. The stimulatory effect of fMet-Leu-Phe is inhibited by prior treatment of the cells with pertussis toxin (not shown). Preincubation with PMA inhibits the ability of fMet-Leu-Phe to stimulate the GTPase defined above. On the other hand, not only does the PMA analog 4-α-PDD not inhibit the effect of fMet-Leu-Phe, but it apparently potentiates it. Though unexplained at present, this effect of 4-α-PDD may be the result of the highly hydrophobic nature of 4-α-PDD [21]. It is worthwhile noting that the basal activity of the GTPase was also inhibited by pretreatment of the cells with PMA.

It is clear from the results described above that preincubation of the neutrophils with PMA increases, under our experimental conditions, the ability of pertussis toxin to ribosylate a protein of $M_r = 41\,000$ that is likely to be the α-subunit of the pertussis toxin substrate, and inhibits the ability of fMet-Leu-Phe to stimulate a GTPase activity presumably due to the latter. In addition, Katada

Table 3

Effect of pretreatment with phorbol esters on the basal and fMet-Leu-Phe stimulated GTPase

Condition[a]	GTPase activity (pmol/min per 10^7 cells)[b]		
	Basal	fMet-Leu-Phe[c]	% increase
Control	3.85 ± 0.08	5.00 ± 0.15	30
+ PMA (50 ng/ml)	2.91 ± 0.08	3.20 ± 0.20	10
+ 4-α-PDD (50 ng/ml)	3.12 ± 0.10	4.56 ± 0.20	46

[a] The cells were incubated with PMA or 4-α-PDD for 3 min before they were homogenized. The control cells have the same concentration of DMSO as the other cells

[b] The values are the mean ± SE of at least 3 separate experiments. All the reactions were carried out for 10 min. The values were calculated as the differences between zero and 10 min

[c] The synthetic peptide fMet-Leu-Phe at 10^{-7} M was added to the homogenates and the reaction was carried out for 10 min

et al. [22] have also demonstrated that the PMA activated protein kinase C was capable of phosphorylating dissociated α, but not intact N_i.

The full elucidation of the biochemical basis of the effects of PMA just described clearly awaits the results of further, and more direct, investigations. As they stand, however, these results do suggest a model that explains the various findings related to the inhibitory activities of phorbol esters. It should be pointed out that it is unlikely that the present observations can be ascribed solely to the limited fusion of granule membrane with the plasma membrane that may have occurred during the preincubation period with PMA.

The results described here are compatible with the hypothesis that PMA, through its effects on the activity of protein kinase C [23], shifts the association/dissociation state of the GTP-binding protein to its oligomeric form. A diagrammatic representation of the events envisioned by this hypothesis is presented in fig.2. α-GDP produced either from the spontaneous dissociation of the GTP-binding protein oligomer or following the activation of the GTPase activity inherent in its α-subunit, is the substrate for protein kinase C. Although the stimulation in the PMA treated cells of the ADP-ribosylation catalyzed by pertussis toxin could be due to an increase in the amount of

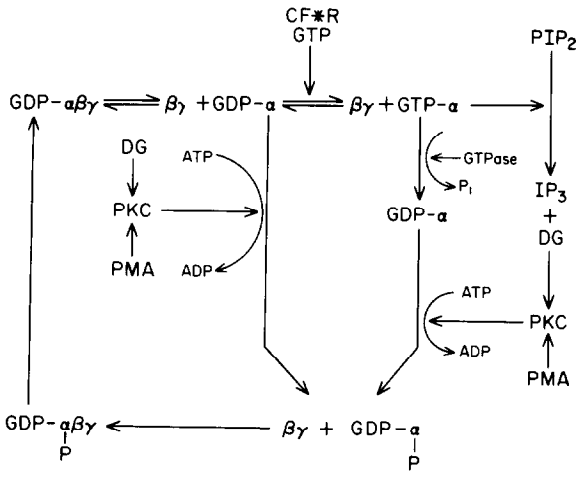

Fig.2. Schematic representation of the interaction of the 'inhibitory' guanine nucleotide-binding protein and protein kinase C in neutrophil activation. CF, chemotactic factor; R, receptor; α-P, phosphorylated state of the α-subunit of N_i.

the substrate and/or the rate of ribosylation, preliminary experiments indicate that it is the former parameter that is altered under our conditions. It remains to be demonstrated whether the amount of the undissociated oligomer (GDP-$\alpha\beta\gamma$), which is the substrate for pertussis toxin, is increased by PMA treatment and/or whether the phosphorylated oligomer is a better substrate than the dephosphorylated counterpart. This model also explains the recent interesting observation by Katada et al. [22] that GTP[S]-α-subunit is not a good substrate for protein kinase C. The increased level of ribosylation following the treatment of intact cells by fMet-Leu-Phe can be ascribed to the concomitant activation of protein kinase C; the decreased ribosylation in homogenates, on the other hand, reflects a dissociation of the GTP-binding protein oligomer that is not followed by reassociation, possibly because of the release of the α-subunit into the suspending media [24]. Furthermore, the heterologous desensitization phenomenon commonly observed in hormonally responsive cells can be easily accounted for by this model. Though speculative in several respects, this model accommodates the results of the above experiments and those concerning the activity of the α-subunit as a substrate for protein kinase C. This model is clearly a working hypothesis, and as such, its appeal lies not so much in the correctness of its details as in its ability to suggest tests of its validity.

In summary, the above results indicate that significant modulation by protein kinase C of signal transduction in calcium mobilizing cells is possible at the level of the activation of the guanine nucleotide-binding regulatory proteins.

ACKNOWLEDGEMENTS

Supported in part by NIH grants AI-13734, AI-09648, AM-31000 and GM-17536.

REFERENCES

[1] Joseph, S.K. (1985) Trends Biochem. Sci. 10, 297–298.
[2] Cockcroft, S. and Gomperts, B.D. (1985) Nature 314, 534–536.
[3] Smith, C.D., Lane, B.C., Kusaka, I., Verghese, M.W. and Snyderman, R. (1985) J. Biol. Chem. 260, 5875–5878.

[4] Wallace, M.A. and Fain, J.N. (1985) J. Biol. Chem. 260, 9527–9530.
[5] Molski, T.F.P., Naccache, P.H., Marsh, M.L., Kermode, J., Becker, E.L. and Sha'afi, R.I. (1984) Biochem. Biophys. Res. Commun. 124, 644–650.
[6] Okajima, F. and Ui, M. (1984) J. Biol. Chem. 259, 13863–13871.
[7] Bokoch, G.M. and Gilman, A.G. (1984) Cell 39, 301–308.
[8] Verghese, M.W., Smith, C.D. and Snyderman, R. (1985) Biochem. Biophys. Res. Commun. 127, 450–457.
[9] Volpi, M., Naccache, P.H., Molski, T.F.P., Shefcyk, J., Huang, C.-K., Marsh, M.L., Munoz, J., Becker, E.L. and Sha'afi, R.I. (1985) Proc. Natl. Acad. Sci. USA 82, 2708–2712.
[10] Brandt, S.J., Dougherty, R.W., Lapetina, E.J. and Niedel, J.E. (1985) Proc. Natl. Acad. Sci. USA 82, 3277–3280.
[11] Goldman, D.W., Chang, F.-H., Gilford, L.A., Goetzl, E.J. and Bourne, H.R. (1985) J. Exp. Med. 162, 145–156.
[12] MacIntyre, D.E., McNicol, A. and Drummond, A.H. (1985) FEBS Lett. 180, 160–164.
[13] Rittenhouse, S.E. and Sasson, J.P. (1985) J. Biol. Chem. 260, 8657–8660.
[14] Zavoico, G.B., Halenda, S.P., Sha'afi, R.I. and Feinstein, M.B. (1985) Proc. Natl. Acad. Sci. USA 82, 3859–3862.
[15] Orellana, S.A., Solski, P.A. and Brown, J.H. (1985) J. Biol. Chem. 260, 5236–5239.
[16] Naccache, P.H., Molski, T.F.P., Borgeat, P., White, J.R. and Sha'afi, R.I. (1985) J. Biol. Chem. 260, 2125–2131.
[17] Schell-Frederick, E. (1984) Cell Calcium 5, 237–251.
[18] Showell, H.J., Williams, D., Becker, E.L., Naccache, P.H. and Sha'afi, R.I. (1979) J. Reticulo. Endothel. Soc. 25, 139–150.
[19] Okajima, F., Katada, T. and Ui, M. (1985) J. Biol. Chem. 260, 6761–6768.
[20] Hyslop, P.A., Oades, Z.G., Jesaitis, A.J., Painter, R.G., Cochrane, C.G. and Sklar, L.A. (1984) FEBS Lett. 166, 165–169.
[21] Jacobson, K., Wenner, C.E., Kemp, G. and Papahadjopoulos, D. (1975) Cancer Res. 35, 2991–2995.
[22] Katada, T., Gilman, A.G., Watanabe, Y., Bauer, S. and Jakobs, K.H. (1985) Eur. J. Biochem. 151, 431–437.
[23] Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, V. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851.
[24] Rodbell, M. (1985) Trends Biochem. Sci. 10, 461–464.