Polymyxin B inhibits phorbol 12-myristate 13-acetate, but not chemotactic factor, induced effects in rabbit neutrophils

Paul H. Naccache, Marshall M. Molski and Ramadan I. Sha'afi

Departments of Pathology and Physiology, University of Connecticut Health Center, Farmington, CT 06032, USA

Received 8 September 1985

The addition of the amphipathic polycationic antibiotic polymyxin B to a suspension of rabbit neutrophils results in inhibition of the agonist (secretion of secondary granules) and antagonist (inhibition of chemotactic factor induced degranulation) properties of phorbol 12-myristate 13-acetate. On the other hand, polymyxin B does not inhibit the degranulation of the neutrophils that is induced by chemotactic factors. These results imply that the role of protein kinase C in the initiation of neutrophil functions in response to the addition of chemotactic factors is less critical than previously thought. In addition, the reversal of the inhibitory properties of phorbol esters by polymyxin B indicates that the former are mediated by the ability of the tumor promoters to activate protein kinase C. These results thus strengthen the hypothesis that protein kinase C plays important roles in the regulation (as contrasted to initiation) of neutrophil functions.

Polymyxin B *Protein kinase C* *Neutrophil*

1. INTRODUCTION

The activation and/or intracellular translocation of protein kinase C figures prominently in most current models of non-muscle cell activation [1]. The evidence implicating this enzyme in the above functions includes its presence in the target tissues, the demonstration of the stimulation of its activity by various agonists, and of the biological activities of phorbol diesters. The actions of the latter compounds are generally thought to result from their ability to bind to and activate protein kinase C. The above evidence, however compelling, is essentially of a correlative nature in the absence of the definition of the biological roles of the substrates of protein kinase C and thus deserves additional probing.

In an effort to examine further the potential involvement of protein kinase C in the activation of the polymorphonuclear neutrophilic leukocytes (neutrophils), we have examined the effects of polymyxin B on the responses of these cells to chemoattractants and phorbol diesters. These studies were prompted by the reports of the inhibitory activity of the antibiotic towards protein kinase C [2–4]. The results to be presented indicate that while polymyxin B is a potent inhibitor of the degranulation of rabbit neutrophils induced by phorbol 12-myristate 13-acetate (PMA), it, in contrast, does not antagonize the secretory activity of the formylated oligopeptides chemoattractants. In addition, pretreatment of the neutrophils with polymyxin B prevents the inhibition of responsiveness towards the chemotactic factor that is induced by PMA. The implication of these results for the role of protein kinase C in the initiation and maintenance of stimulated neutrophil functions will be discussed.

2. MATERIALS AND METHODS

Rabbit peritoneal neutrophils obtained 4 or 16 h after the injection of sterile glycogen solutions were used throughout these experiments. They were collected, handled and resuspended in Hanks' balanced salt solution containing no added magnesium or protein as reported [5].

Neutrophil degranulation was monitored by

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

following the extracellular release of *N*-acetyl-β-glucosaminidase, lysozyme and lactate dehydrogenase. The release of the latter, an index of cell viability, did not differ significantly between the untreated control cell samples and the treated ones, and did not exceed 7% of the total cell content of this enzyme. Unless otherwise noted, the characteristics of the release of the 2 granular enzymes as induced by fMet-Leu-Phe closely paralleled each other.

Polymyxin B was purchased from Sigma (St. Louis, MO), fMet-Leu-Phe from Peninsula Labs. (San Carolos, CA) and PMA from CMC Cancer Chemicals (Brewster, NY).

3. RESULTS AND DISCUSSION

The cyclic polycationic peptide antibiotic polymyxin B has recently been shown to be a potent inhibitor of the calcium- and phospholipid-dependent protein kinase generally referred to as protein kinase C [2,3]. Polymyxin B has also been shown to inhibit the secretion of amylase from isolated pancreatic acini [3] and the stimulation of protein kinase C activity and proliferation induced by phorbol esters in B lymphocytes [4]. As protein kinase C had been implicated in the mechanism of initiation and regulation of neutrophil function by various agents including chemoattractants and phorbol esters [6–12], it was thus of immediate relevance to examine the effects of polymyxin B on neutrophil degranulation induced by these 2 classes of agonists.

The addition of polymyxin B prior to that of PMA reduces in a concentration-dependent manner the secretion of lysozyme from the neutrophils (fig.1). The IC_{50} of the inhibition of lysozyme release by polymyxin B, obtained at 100 ng/ml PMA, is about 0.1 mM. Note that the basal level of lysozyme release, that is observed following the 30 min incubation in the absence of any secretagogue, is also reduced in the presence of the antibiotic. In contrast (fig.2), neutrophil degranulation induced by the formylated peptide chemoattractant fMet-Leu-Phe is not inhibited by polymyxin B. In fact, the antibiotic potentiates slightly, though consistently, the release of *N*-acetyl-β-glucosaminidate from the cells while it leaves unaffected that of lysozyme. Preliminary experiments have likewise shown that the secretory responses to

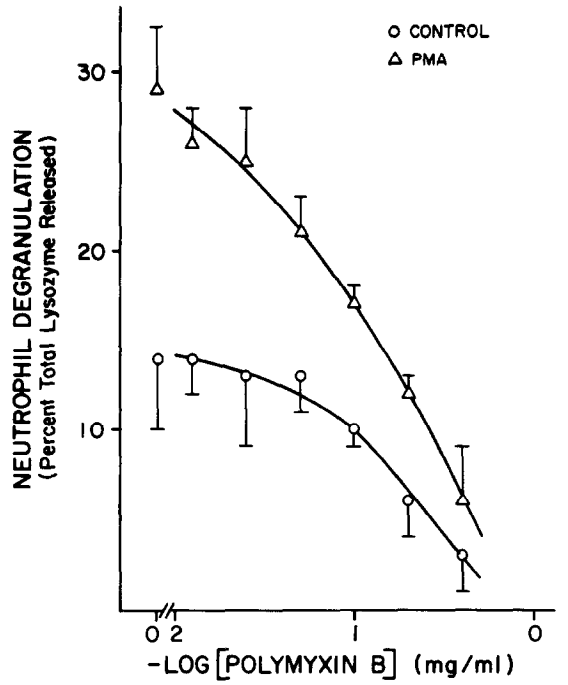

Fig.1. Concentration dependence of the inhibitory effect of polymyxin B on the secretion of lysozyme induced by PMA in rabbit neutrophils. The concentration of PMA was 100 ng/ml; the incubation time with the phorbol ester, 30 min and the preincubation time with polymyxin B, 10 min. The data represent the mean ± SE of 3 experiments each carried out in duplicates.

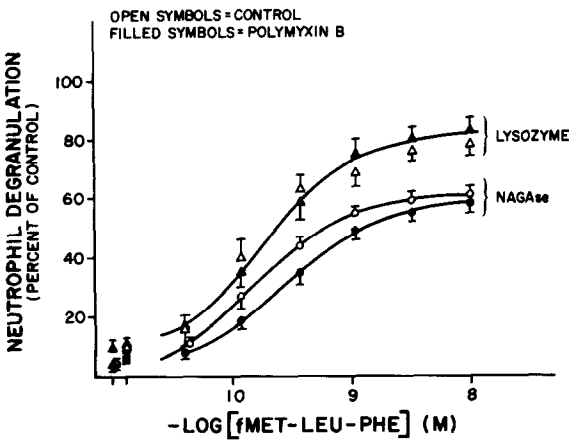

Fig.2. Effect of polymyxin B on the degranulation of rabbit neutrophils as induced by fMet-Leu-Phe and cytochalasin B. The concentration of cytochalasin B was 2.5 μg/ml, and the incubation times with the chemotactic factor and the antibiotic 5 and 10 min, respectively. The data represent the mean ± SE of at least 7 experiments each carried out in duplicates.

leukotriene B_4 and platelet activating factor are not inhibited by preincubation with polymyxin B.

Phorbol esters such as PMA have recently been shown to inhibit the responses of a variety of cells if added a few minutes before the respective agonists. In the neutrophils, PMA inhibits the ability of several chemoattractants to induce secretory and oxidative responses and to raise the cytoplasmic levels of calcium [9,10,12]. The mechanism by which PMA expresses its antagonist activity is not known. It is thought however to depend on the ability of the phorbol ester to activate protein kinase C, as analogs that lack tumorigenic and protein kinase C stimulatory activities do not inhibit cell activation. We have thus taken advantage of the demonstrated inhibitory activity of polymyxin B towards protein kinase C to probe further into the mechanism of inhibition of cell function by phorbol esters. The experiment whose results are shown in fig.3 was designed to examine the effect of polymyxin B on the inhibition of

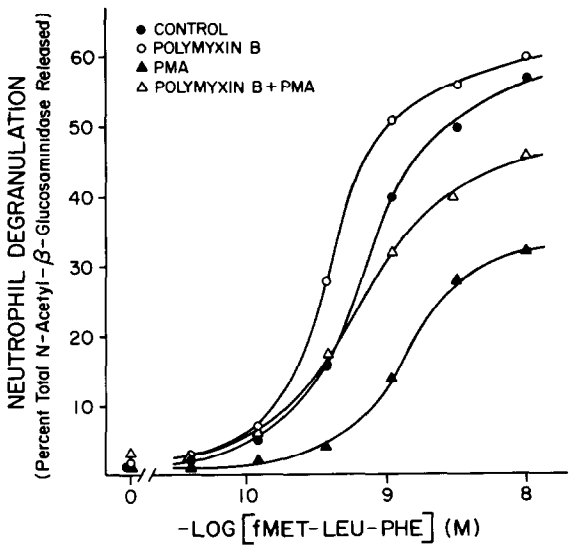

Fig.3. Effect of polymyxin B on the inhibition by PMA of the secretory response of rabbit neutrophils to the addition of fMet-Leu-Phe and cytochalasin B. The cells were preincubated with polymyxin B for 10 min; PMA (50 ng/ml) was added to the desired suspensions and the incubation carried out for an additional 3 min. The cells were then transferred to tubes containing serially diluted concentrations of fMet-Leu-Phe and 2.5 μg/ml cytochalasin B. The data are from a single experiment representative of at least 3 others similarly designed.

neutrophil secretory responsiveness towards fMet-Leu-Phe. PMA can clearly be seen to diminish the secretory response of the cells induced by the chemotactic factor (decreased maximal release and shift in the ED_{50}). Polymyxin B, by itself, and as illustrated in fig.2, slightly potentiates the degranulation response. When added before the phorbol ester, polymyxin B can be seen to prevent in great part the inhibitory action of the phorbol ester. These results support the contention that the inhibition of cell functions by PMA that have been documented in a variety of cell systems do indeed stem from the ability of these compounds to interact with protein kinase C.

There are several possible interpretations to the data presented above. The most literal would be that protein kinase C, though present in the neutrophils [13,14] and activated upon the addition of chemotactic factors [7,8,11] is not necessary for the degranulation response of the cells that is evoked by chemoattractants. The function of the kinase would, according to this interpretation, still have to be defined. An alternative explanation is that there are different classes of protein kinase C and that polymyxin B does not affect the subset that is utilized by fMet-Leu-Phe. A third possibility is that polymyxin B is a weak agonist for protein kinase C, capable of blocking the binding of PMA to this enzyme but not of activating it sufficiently to produce a detectable biological response. This interpretation would explain the slight potentiation of the secretion of *N*-acetyl-β-glucosaminidase that was observed when fMet-Leu-Phe was the secretagogue. Firstly, it should be pointed out that one cannot completely rule out the possibility that the increase in calcium induced by chemotactic factors overcomes the inhibition of protein kinase C by polymyxin B. This hypothesis is however weakened significantly by the in vitro finding that the antibiotic antagonizes the activation of protein kinase C in the presence of 0.5 mM calcium [3]. Sorting out these alternative hypotheses requires much additional experimentation. However, as they stand, the present results indicate that caution needs to be exercised regarding the possible physiological role of protein kinase C in the physiological activation of the neutrophils.

With respect to the commonly observed inhibition of cell function by phorbol esters that has been observed in a wide variety of systems (e.g.

[15–17]), including the chemotactic factor stimulated neutrophils, the present results strongly suggest that this apparent negative feedback is mediated by the activation of protein kinase C. The latter may result in the phosphorylation of the guanine nucleotide binding proteins that have recently been implicated in signal transmission in calcium-dependent cells such as the neutrophils [18–20], the mast cells [21] and the platelets [22], and thereby interrupt the transfer of information across the plasma membrane.

ACKNOWLEDGEMENT

This work was supported in part by NIH grants AM-31000 and AI-13734.

REFERENCES

[1] Nishizuka, Y. (1984) Nature 308, 693–696.
[2] Wise, B.C., Glass, D.B., Jen Chou, C.-K., Raynor, R.L., Katoh, N., Schatzman, R.C., Turner, R.S., Kibler, R.F. and Kuo, J.F. (1982) J. Biol. Chem. 257, 8489–8495.
[3] Wrenn, R.W. and Wooten, M.W. (1984) Life Sci. 35, 267–276.
[4] Nel, A.E., Wooten, M.W., Goldschmidt-Clermont, P.J., Miller, P.J., Stevenson, H.C. and Galbraith, R.M. (1985) Biochem. Biophys. Res. Commun. 128, 1364–1372.
[5] Showell, H.J., Williams, D., Becker, E.L., Naccache, P.H. and Sha'afi, R.I. (1979) J. Reticuloendothel. Soc. 25, 1139–1150.
[6] Sha'afi, R.I., White, J.R., Molski, T.F.P., Schefcyk, J., Volpi, M., Naccache, P.H. and Feinstein, M.B. (1983) Biochem. Biophys. Res. Commun. 114, 638–645.
[7] Andrews, P. and Babior, B.M. (1984) Blood 64, 883–890.
[8] White, J.R., Huang, C.-K., Hill, J., Naccache, P.H., Becker, E.L. and Sha'afi, R.I. (1984) J. Biol. Chem. 259, 44–50.
[9] Naccache, P.H., Molski, T.F.P., Borgeat, P., White, J.R. and Sha'afi, R.I. (1985) J. Biol. Chem. 260, 2125–2131.
[10] Schell-Frederick, E. (1984) Cell Calcium 5, 237–251.
[11] Schneider, C., Zanetti, M. and Romeo, D. (1981) FEBS Lett. 127, 4–8.
[12] Gennaro, R., Pozzan, T. and Romeo, D. (1984) Proc. Natl. Acad. Sci. USA 81, 1416–1420.
[13] Huang, C.-K., Hill, J.M., Bormann, B.J., Mackin, W.M. and Becker, E.L. (1983) Biochim. Biophys. Acta 760, 126–135.
[14] Helfman, D.M., Applebaum, B.D., Vogler, W.R. and Kuo, J.F. (1983) Biochem. Biophys. Res. Commun. 111, 847–853.
[15] Garte, S.J. and Belman, S. (1980) Nature 284, 171–173.
[16] Zavoico, G.B., Halenda, S.P., Sha'afi, R.I. and Feinstein, M.B. (1985) Proc. Natl. Acad. Sci. USA 83, 3859–3862.
[17] Sagi-Eisenberg, R., Lieman, H. and Pecht, I. (1985) Nature 313, 59–60.
[18] Molski, T.F.P., Naccache, P.H., Marsh, M.L., Kermode, J.C., Becker, E.L. and Sha'afi, R.I. (1984) Biochem. Biophys. Res. Commun. 124, 644–650.
[19] Okajima, F. and Ui, M. (1984) J. Biol. Chem. 259, 13863–13871.
[20] Bokoch, G.M. and Gilman, A.G. (1984) Cell 39, 301–308.
[21] Gomperts, B.D. (1983) Nature 306, 64–66.
[22] Haslam, R.J. and Davidson, M.M.L. (1984) FEBS Lett. 174, 90–95.