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THE INHIBITION OF NEUTROPHIL RESPONSIVENESS CAUSED BY PHORBOL ESTERS IS BLOCKED BY THE PROTEIN KINASE C INHIBITOR H7

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SUMMARY: The relationship between the inhibition of neutrophil responsiveness to chemoattractants caused by preincubation with phorbol esters and the activation of protein kinase C was investigated using the protein kinase antagonist H7. The latter compound was found to inhibit the phosphorylation of the 50 kDa protein kinase C substrate stimulated by phorbol 12-myristate 13-acetate (PMA). On the other hand, H7 was found not to affect the quin2 and secretory responses of the neutrophils to fMet-Leu-Phe and leukotriene B_4. In addition, pretreatment of the cells with H7 blocked the ability of PMA to inhibit the latter two responses to the addition of the chemoattractants. Taken together, these results provide strong evidence for the involvement of protein kinase C in the inhibition of neutrophil - and probably also other cells - responsiveness brought about by preincubation with phorbol esters. Additionally, they invite a reevaluation of the role of protein kinase C in the excitation-response coupling sequence of these cells directed more towards a negative, modulatory, role than that of a critical element in its initiation.

The activation and/or translocation of protein kinase C figures prominently in most current models of non-muscle cell activation (Nishizuka, 1984), including that of the polymorphonuclear leukocytes (neutrophils) (Sha'afi et al., 1983; White et al., 1984; Wolfson et al., 1985; Gennaro et al., 1986). The evidence implicating this enzyme in stimulus-response coupling includes, among others, its presence in the target tissues, the demonstration of the stimulation of its activity by various agonists, and the biological activities (both agonist and antagonist) of phorbol esters (Nishizuka, 1984). The latter compounds have been shown to bind to, and activate protein kinase C in intact cells and in purified preparation of this enzyme (Castagna et al., 1982; Niedel et al., 1983),

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and their biological effects are generally thought to be mediated by the stimulation of the activity of protein kinase C.

Phorbol esters have recently been found to inhibit the functional responsiveness of various cell types stimulated by receptor dependent agonists (e.g., Labarca et al., 1984; McIntyre et al., 1985; Rittenhouse and Sasson, 1985; Zavoico et al., 1985; Schell-Frederick, 1984; Naccache et al., 1985; Sagi-Eisenberg et al., 1985; Vicentini et al., 1985). These results have raised the possibility that one of the major physiological functions of the stimulation of protein kinase C is to generate negative, or termination signals. Though the exact mechanism of cell inhibition by phorbol esters remains to be delineated, the presently available data suggest that the activation of protein kinase C may result in the phosphorylation, and inactivation, of one or more of the components of the guanine nucleotide binding proteins (alpha, or a closely analogous protein) (Katada et al., 1985). The latter have recently been postulated to mediate the receptor dependent stimulation of the hydrolysis of the polyphosphoinositides - an event that appears central to the excitation-response coupling sequence (Berridge, 1984) - in several cell types and in particular in the neutrophils (Smith et al., 1985; Cockroft and Gomperts, 1985).

We undertook the present studies to test the hypothesis that the activation of protein kinase C underlies the impairment of neutrophil responsiveness that is induced by preincubation with phorbol esters. The results to be presented show that the addition of the protein kinase C inhibitor H7 (Kawamoto et al., 1984; Hidaka et al., 1984; Inagaki et al., 1984) to a suspension of rabbit neutrophils blocks the agonist (phosphorylation of the 50 kDa protein) and antagonist (inhibition of chemotactic factor induced rise in the intracellular concentration of calcium and degranulation) properties of phorbol 12-myristate 13-acetate.

MATERIALS AND METHODS

Rabbit peritoneal neutrophils collected from 4 or 16 hour exudates were used throughout these experiments. They were washed twice and resuspended in Hanks' balanced salt solution buffered to pH 7.3 with 10 mM HEPES in the absence of magnesium or protein as previously described (Showell et al., 1979).

Neutrophil degranulation was monitored by following the extracellular appearance of N-acetyl-beta-glucosaminidase as previously described (Showell et al., 1979). Lactate dehydrogenase release, a marker of cell damage, did not differ in control, untreated cells, or in the experimentally manipulated cells and in either case did not exceed 6% of the total cell contents.

The intracellular levels of free calcium were inferred from the measurement of the fluorescence of the calcium indicator quin2. The loading and recording conditions were as previously described (Naccache et al., 1985).

To determine the effect of H7 on the ability of PMA to induce protein phosphorylation, cells (10^8 cells/ml) were incubated with [^{32}P] orthophosphate (1 mCi/ml) as described previously (White et al., 1984) with slight modification. The cells were incubated for 2 hrs at 37°C and 1 mg/ml of ovalbumin was added to the incubation buffer. At the end of the incubation period the cells were washed 2 times and resuspended to give a final concentration of 3×10^7 cells/ml. Cells were divided in half and one half was treated with H7 (25 µm). After incubation at 37°C for 5 min, both control and the H7 treated cells were stimulated with PMA (0.1 µg/ml). The reaction was stopped at various time points by removing an aliquot of the cell suspension (50 µl), mixed with 50 µl of stopping solution (White et al., 1984). The samples were then subjected to SDS-polyacrylamide gel electrophoresis and autoradiographed as described (White et al., 1984). The density of the autoradiography band of Mr 50,000 protein were measured by densitometric scanning (White et al., 1984).

Phorbol 12-myristate 13-acetate (PMA) was purchased from Calbiochem (La Jolla, CA), formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe) from Peninsula Labs. (San Carlos, CA), cytochalasin B from Aldrich Chem. Co. (Milwaukee, WI) and 1-(5-isoquinoline-sulfonyl)-2-methyl piperazine (H7) from Seikagaku America, Inc. (St. Petersburg, FL). Leukotriene B_4 was a generous gift of Dr. P. Borgeat, Groupe de Recherches sur les Leucotrienes, CHUL, Ste Foy, Quebec. All other reagents were reagent grade.

RESULTS AND DISCUSSION

The spectrum of the biological activities of phorbol esters encompasses both stimulatory and inhibitory effects on the target cells depending on the experimental conditions used (for a recent review, see Drummond and Macintyre, 1985). This study was thus designed to define the effects of the protein kinase C inhibitor H7 towards both the agonist and antagonist activities of PMA in rabbit neutrophils.

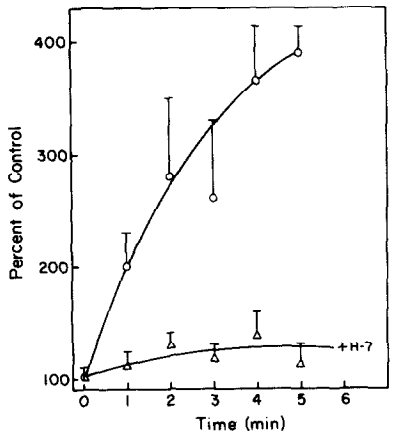

Figure 1. Effect of H7 on the ability of PMA to induce the phosphorylation of the 50 kDa protein in rabbit neutrophils. The cell suspensions were loaded with ^{32}P as described in Materials and Methods. H7 was used at a concentration of 2.5×10^{-5} M and the cells were incubated with it for 5 minutes. PMA was added to the cells at a concentration of 100 ng/ml. The reactions were stopped at the indicated times by withdrawing samples and adding them to tubes containing stopping solution. Mean ± SEM of three experiments.

The results presented in Figure 1 demonstrate that the phosphorylation that is induced by PMA of a neutrophil protein of apparent molecular weight of about 50 kDa is inhibited by H7. This protein has previously been shown to be one of the major phosphorylation substrates in neutrophils stimulated by PMA and also of homogenates exposed to phosphatidyl serine and calcium (i.e., conditions designed to specifically activate protein kinase C) (Schneider et al., 1981; Andrews and Babior, 1983, 1984; White et al., 1984; Huang et al., 1983). In addition, the tryptic pattern of the 50 kDa protein phosphorylated either by exposure of whole cells to PMA (or fMet-Leu-Phe) or of homogenates to phosphatidyl serine and calcium was found to be similar enough to suggest that the PMA induced phosphorylation of the 50 kDa protein is mediated by protein kinase C (White et al., 1984). The data presented in Figure 1 thus indicate that H7 does indeed inhibit the protein kinase C of the neutrophils that is activated by PMA. It should be pointed out, however, that H7 may also affect the activity of other protein kinases including the cyclic AMP dependent one. Based on the evidence just presented,

however, this potential lack of specificity of H7 should not detract from the conclusion reached.

We have examined next the effects of pretreatment of the cells with H7 on the ability of PMA to prevent the quin2 and secretory responses normally evoked by fMet-Leu-Phe and leukotriene B_4.

The results presented in Figures 2 and 3 confirm first the previously reported inhibition by PMA of the increase in quin2 fluorescence induced by fMet-Leu-Phe and leukotriene B_4 (Sha'afi et al., 1983; Schell-Frederick, 1984; Naccache et al., 1985; Lagast et al., 1984). The increase in quin2 fluorescence reflects most probably the rise in free calcium

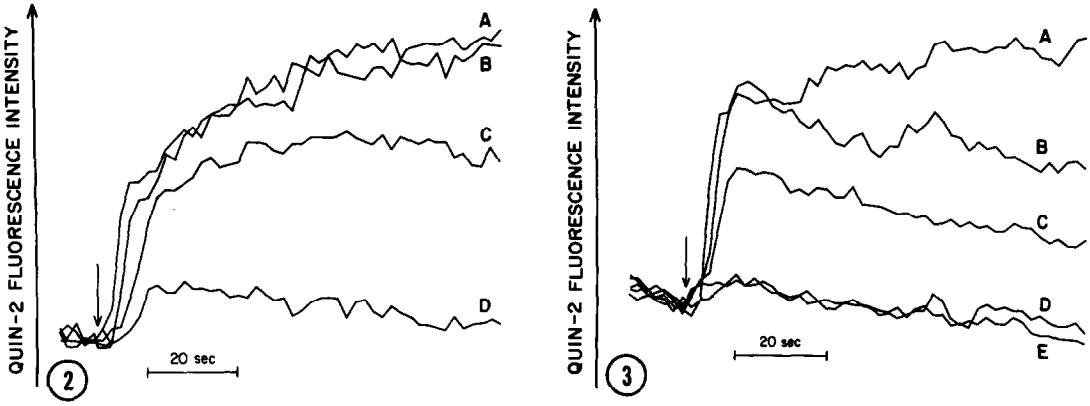

Figure 2. Effect of H7 on the rise in quin2 fluorescence induced by fMet-Leu-Phe and on the inhibition of that response caused by preincubation with PMA. When used, H7 (2.5×10^{-5} M) was added to the cells 5 min prior to the other stimuli, and PMA (50 ng/ml) 3 min prior to the addition of fMet-Leu-Phe (4×10^{-10} M). The conditions are as follows: A. fMet-Leu-Phe alone; B. fMet-Leu-Phe and H7; C. fMet-Leu-Phe added to cells first treated with H7 and then with PMA; D. fMet-Leu-Phe added to cells pretreated with PMA. The tracings are from a single experiment representative of at least three others.

Figure 3. Effect of H7 on the rise in quin2 fluorescence induced by leukotriene B_4 and on the inhibition of that response caused by preincubation with PMA. The concentration of leukotriene B_4 was 7.2×10^{-8} M, that of H7 2.5×10^{-5} M, and that of PMA either 5 or 50 ng/ml. The conditions are as follows: A. leukotriene B_4 alone; B. leukotriene B_4 added to cells first treated with H7 and then with 5 ng/ml PMA; C. leukotriene B_4 added to cells first exposed to H7 and then to 50 ng/ml PMA; D. leukotriene B_4 addded to cells treated with 50 ng/ml PMA; E. leukotriene B_4 added to cells treated with 5 ng/ml PMA. H7, on its own was found not to affect the response to leukotriene B_4, and the tracing was omitted to clarify the figure. The tracings are from a single experiment representative of at least three others.

that is thought to mediate, at least in part, the activation of the neutrophils by chemoattractants (Sha'afi and Naccache, 1981) and which has also been detected using radioisotopic methods (Naccache et al., 1977; Petroski et al., 1979). Of direct relevance to present studies are the two observations that H7 is itself without effect on the response of the cells to the chemoattractants, and that H7 blocks to a significant extent the PMA induced inhibition of the quin2 response to these two agonists.

A similar pattern emerges in the degranulation assay (Table I). H7, by itself, does not inhibit the secretory response to fMet-Leu-Phe. In fact, a small potentiation by H7 of the exocytotic response to the agonist can be observed, an effect reminiscent of that previously observed in the neutrophils with polymyxin B (Naccache et al., 1985b), another compound with anti-protein kinase C activity, and with H7 in the platelets (Inagaki et al., 1984) and the mast cells (Okano et al., 1985). In addition, the inhibition by PMA of the release of N-acetyl-

TABLE I

EFFECT OF H7 ON THE BASAL AND fMET-LEU-PHE STIMULATED RELEASE OF N-ACETYL-BETA-GLUCOSAMINIDASE IN CONTROL AND PMA TREATED CELLS

EXP. CONDITIONS	NAGase RELEASE (% of total)		
	Basal	+ fMet-Leu-Phe	
		0.4 nM	0.8 nm
No additions[1]	7 ± 4	48 ± 4	58 ± 6
H7 (2.5×10^{-5} M)[2]	7 ± 5	55 ± 8	65 ± 10
PMA (50 ng/ml)[3]	10 ± 6	14 ± 3	24 ± 5
H7 + PMA[4]	13 ± 8	35 ± 4	55 ± 12

[1]Neutrophil degranulation was measured as described in Materials and Methods. Mean ± SEM of three separate experiments.

[2]The cells were incubated with H7 for 5 minutes before being transferred to the degranulation assay.

[3]The cells were incubated with PMA for 3 minutes before being transferred to the degranulation assay.

[4]The cells were first incubated with H7 for 5 minutes and then with PMA for 3 minutes.

beta-glucosaminidase produced by fMet-Leu-Phe can be seen to be significantly blocked by pretreatment of the cells with 2.5 x 10^{-5} M H7.

A dose response of the blockage by H7 of the inhibitory activity of PMA towards neutrophil degranulation produced by fMet-Leu-Phe is presented in Figure 4. These results clearly show that the effect of H7 is concentration dependent, and is detectable at concentrations equal to, or larger than 3 x 10^{-6} M, i.e., at concentrations similar to its Ki toward protein kinase C (Hidaka et al., 1984). An essentially complete blockage of the inhibitory effect of PMA is seen at 2.5 x 10^{-5} M H7 in the presence of 8 x 10^{-10} M fMet-Leu-Phe, while at 4 x 10^{-10} M fMet-Leu-Phe, the inhibition by the phorbol ester is reduced from 90 ± 2% in the absence of H7 to 51 ± 3% in its presence. Further experiments are required to define the kinetic parameters of the antagonism by H7 of the inhibitory properties of PMA and to determine the nature (competitive vs. non-competitive, etc.) of these interactions.

The results presented above make three separate, though interrelated, points.

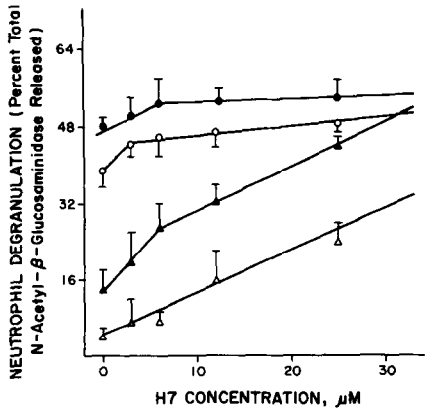

Figure 4. Effect of H7 on the inhibition of the fMet-Leu-Phe induced neutrophil degranulation that is caused by pretreatment with PMA. Neutrophil degranulation was measured as described in Materials and Methods. The concentration of PMA was 100 ng/ml and the preincubation time 3 minutes. The circles represent the data obtained from cells that had not been exposed to the phorbol ester; the triangles from those that had been preincubated with PMA. The concentrations of fMet-Leu-Phe were as follows: filled symbols: 8 x 10^{-10} M; open symbols: 4 x 10^{-10} M. The data represent the mean ± SEM of three experiments.

1. The ability of PMA to stimulate protein phosphorylation is inhibited by H7. Moreover, Gerard et al. (1986) have also very recently demonstrated that the stimulation by PMA of the oxidative metabolism of human neutrophils could be inhibited by an analog of H7, compound C-1. These results strengthen the hypothesis that the agonist properties of PMA towards the neutrophils are based on its ability to induce an intracellular translocation and to activate protein kinase C, a conclusion that was previously based mostly on correlative studies with structural analogs of the phorbol esters with varying protein kinase C potentiating activities.

2. The quin2 and secretory responses of the neutrophils to fMet-Leu-Phe and leukotriene B_4 are not inhibited by H7. Similar results were obtained with polymyxin B in the same assays (Naccache et al., 1985b) and with the H7 analog, compound C-1, using the oxidative metabolism of these cells as an index of cell activation (Gerard et al., 1986). These results may be related to the previously observed inability of fMet-Leu-Phe to induce in the neutrophils a translocation of cytosolic protein kinase C to a particulate fraction (McPhail et al., 1984), in contrast to the well documented effect of PMA in several cell types (Kraft and Anderson, 1981; Wolfson et al., 1985; White et al., 1985; Gennaro et al., 1985; Wolf et al., 1985). Two potential interpretations exist: 1) the stimulus response coupling sequence initiated by fMet-Leu-Phe, $C5_a$, and leukotriene B_4, does not require the activation of protein kinase C, or 2) the occupation of surface receptors by the respective agonists results in the activation of a subset of protein kinase C that is protected from inactivation by H7, C-1 and polymyxin B. Though the first explanation appears more likely on the basis of the presently available data, additional experimentation is clearly required for an unambiguous differentiating between these two alternatives.

3. Possibly the most significant contribution of the present studies is the finding that H7 can block the inhibitory effects of

PMA in both the quin2 and the secretion assays. The data presented above provide an alternative approach to that previously utilized to correlate the ability of PMA to activate protein kinase C and to inhibit neutrophil responsiveness to chemoattractants. Taken together with the demonstrated anti-protein kinase C activity of H7 (see also Hidaka et al., 1984), the present results strongly imply that the inhibitory activities of phorbol esters are indeed mediated by protein kinase C. One of the immediate implications of this interpretation is that the physiological function of this enzyme in the excitation-response coupling sequence may be of a negative, modulatory nature, and that, in contrast to earlier views, protein kinase C may not play any critical role in the initiation of cell responsiveness.

Finally, the precise site at which PMA interrupts the excitation response coupling sequence in the neutrophils as well as in other cells is still to be defined. Recent studies by Katada et al. (1985) have demonstrated that the alpha subunit of the G_i component of the guanine nucleotide binding proteins is a substrate for protein kinase C which, by phosphorylating it, reduces its biological activities. G_i, or a closely analogous protein, has been implicated in signal transmission in calcium mobilizing systems (Gomperts, 1983; Haslan and Davidson, 1984; Molski et al., 1984; Okajima et al., 1984; Bokoch and Gilman, 1984; Nakamura et al., 1984; Volpi et al., 1985; Smith et al., 1985). In addition, we have recently observed that pretreatment of the neutrophils with PMA results in an accumulation, in a presumably inactive state, of the substrate for pertussis toxin, i.e., the relevant GTP binding protein (Matsumoto et al., 1986). It will thus be of direct relevance to examine the effects of H7 on the PMA induced increases in the levels of the substrate of pertussis toxin in the hope that this will help determine whether this particular event is the one responsible for the inhibition of cell responsiveness by phorbol esters.

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