Vol. 167, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1990 Pages 168-173

INHIBITION OF PROTEIN KINASE C BY H-7 POTENTIATES THE RELEASE OF OLEIC, LINOLEIC AND ARACHIDONIC ACIDS IN A23187-STIMULATED HUMAN NEUTROPHILS

Julie Conquer and Vhundi G. Mahadevappa[1]

Cell Biology Research Group
Department of Nutritional Sciences
University of Guelph
Guelph, Ontario, Canada N1G 2W1

Received December 28, 1989

SUMMARY: This present report describes the effect of H-7, a protein kinase C inhibitor, on the release of oleic, linoleic and arachidonic acids in A23187-stimulated neutrophils. Surprisingly, the inhibitor potentiated the release of all three unsaturated fatty acids in neutrophils stimulated with A23187 alone. In contrast, released oleic acid, linoleic acid and arachidonic acid in phorbol 12-myristate 13-acetate-primed neutrophils were attenuated by 35, 47 and 33%, respectively, in the presence of H-7 (300 μM). Phorbol 12-myristate 13-acetate (PMA) had no effect on A23187-stimulated release of saturated fatty acids. Both PMA and H-7 when used alone had no effect on the release of saturated or unsaturated fatty acids. We, therefore, conclude that H-7 may have effects other than inhibiting PMA-primed responses including superoxide generation, degranulation and arachidonic acid release in human neutrophils. © 1990 Academic Press, Inc.

Protein kinase C is a ubiquitous enzyme involved in the transduction of biological signals (1). Tumour promoting phorbol esters such as PMA activate PKC *in vitro* by substituting for diacyl glycerol (DAG), which allows the activation of PKC to occur at fairly low intracellular Ca^{++} concentrations. The addition of PMA to resting neutrophils results in the translocation of PKC from the cytosol to the particulate fraction (membranes) and the subsequent activation of the kinase C (2). Protein kinase C is known to be involved in the phosphorylation of 47 and 80 KD proteins (3,4). Furthermore, several recent studies indicate that PKC may play a predominant role in phospholipase A_2-mediated arachidonic acid release in several cell systems including neutrophils (5-8).

Several inhibitors including 1-(5-isoquinolinesulfonyl)-2-methylpiperazine hydrochloride (H-7) have been used to determine whether or not PKC is involved in various cellular responses such as superoxide production, degranulation, arachidonic acid release and eicosanoid synthesis (6,9-14). Many of these inhibitors appear to have effects other than inhibiting PMA-primed responses (13). Recently it has been shown that H-7 (30 μM) causes a depletion of normal active

[1]To whom correspondence should be addressed.

0006-291X/90 $1.50
Copyright © 1990 by Academic Press, Inc.
All rights of reproduction in any form reserved.

microfilament bundles in 3T3 cells (15). Of the isoquinolinesulfonamides (H-7, H-8, H-9), H-7 appears to be the most potent inhibitor of PKC with a Km of 6 μM (9).

In this communication, we report the effect of H-7 on A23187-stimulated release of arachidonic acid and other unsaturated fatty acids (oleic and linoleic acids) as well as its effects on PMA-primed synergism in regards to the release of oleic, linoleic and arachidonic acids (OA, LA, AA). Our results suggest that H-7 potentiates the phospholipase A_2-like activity in A23187-stimulated neutrophils although the inhibitor attenuates PMA-primed synergism in enhancing the phospholipase A_2 activity.

MATERIALS AND METHODS

Standard fatty acids, A23187 and PMA were obtained from Sigma Chemical Co. (St. Louis, MO). Ficoll-Paque and dextran T-500 were obtained from Pharmacia (Quebec, Canada). Fatty acid free albumin was purchased from Boehringer Mannheim Canada Ltd. (Quebec, Canada). Precoated thin layer silica gel H60 plates were from E. Merck (Darmstadt, FRG). 3-Amino-1-(3-triflouromethylphenyl)-2-pyrazoline hydrochloride (BW755C, a dual inhibitor of cyclooxygenase and lipoxygenase) was generously donated by the Wellcome Research Laboratories, Inc. (Beckenham, U.K.). Protein kinase C inhibitor H-7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazine hydrochloride] was purchased from Seikagau America, Inc. (St. Petersburg, FL).

Isolation of Human Neutrophils

Human neutrophils were isolated essentially as described previously from our laboratory with slight modifications (16). Neutrophil suspensions were prepared in an appropriate volume of HBSS buffer containing 1 mM $CaCl_2$ and 0.1% albumin so as to obtain approximately 2 x 10^7 cells/ml.

Stimulation of Neutrophils

Neutrophil suspensions in HBSS buffer containing 1 mM $CaCl_2$ and 0.1% albumin (2 x 10^7 cells/ml) were primed with PMA (50 nM) for 20 sec and then stimulated with A23187 (3 μM) for 5 min in the presence of BW755C (80 μM), a dual inhibitor of cyclooxygenase/lipoxygenase(s). In separate experiments, neutrophils were stimulated with A23187 or A23187 plus PMA in the presence of H-7, a protein kinase C inhibitor. Reactions were immediately terminated by the addition of 3.75 ml chloroform:methanol (1:2, v/v), followed by 1.25 ml chloroform and 1.25 ml water (17).

Analyses of Released Fatty Acids

Released fatty acids from lipid extracts were fractionated on silica gel H60 thin layer plates using heptane:isopropylether:acetic acid (60:40:3, v/v/v) as the developing solvent system. The fatty acid band was visualized with 2'7'-dichlorofluorescein and identified with the aid of a standard mixture containing both saturated and unsaturated fatty acids. Corresponding free fatty acid bands were immediately scraped off the plate into screw cap glass tubes and methylated with 6% sulphuric acid in methanol for 2 h at 80°C in the presence of heptadecanoic acid (17:0), an internal standard. Following acid catalyzed methylation, fatty acid methyl esters were extracted with petroleum ether and dried under oxygen-free nitrogen. Reconstituted fatty acid methyl esters were analyzed by gas liquid chromatography (GLC) on a fused silica megabore column under isothermal conditions as previously described from our laboratory (16). The area and the corresponding amount of heptadecanoic acid (17:0) were used to determine the absolute amount of individual free fatty acids in the lipid extracts of activated neutrophils.

RESULTS AND DISCUSSION

In general agreement with previous reports (16,18-20), A23187 alone stimulated a highly selective release of arachidonic acid from the sn-2 position of neutrophil phosphoglycerides relative to other unsaturated fatty acids (data not shown). Our results also indicate that the amount of arachidonic acid released from PMA-primed and A23187-stimulated neutrophils was approximately three times greater than that found in neutrophils stimulated with A23187 alone (6.96 vs 2.17 nmoles per 2 x 10^7 neutrophils) (Table 1). These results are in general agreement with previous reports in which it has been shown that PMA, a potent activator of PKC, synergizes the stimulus-induced phospholipase A_2-like activity that releases arachidonic acid for eicosanoid biosynthesis (5,18,21,22). However, the results reported in this study further indicate that PMA also potentiates the release of other unsaturated fatty acids such as oleic acid (18:1, n9) and linoleic acid (18:2, n6) with no apparent effect on saturated fatty acids in A23187-stimulated neutrophils. These findings imply that PMA, which causes the translocation of PKC from cytosol to the membrane, primes the activation of a nonspecific phospholipase A_2 (PLA_2) or modulates the specificity of arachidonic acid-specific PLA_2 so as to release all three unsaturated fatty acids from the sn-2 position of phosphoglycerides in A23187-stimulated neutrophils. The nonspecific PLA_2 has been reported to be associated with azurophilic granules in human neutrophils (23). However, PMA alone does not cause the activation of PLA_2 (specific or non-specific) despite its ability to directly activate PKC (5,6,8,21,22).

As shown in Figure 1, the protein kinase C inhibitor, H-7, exhibits two types of responses depending upon the stimulus used. For example, the inhibitor significantly enhanced the A23187-stimulated release of all three unsaturated fatty acids (Fig. 1 and Table 2). Generally, we observed

Table 1. Effect of Phorbol 12-Myristate 13-Acetate (PMA) on A23187-Stimulated Release of Unsaturated Fatty Acids in Human Neutrophils

	Agonists	
Fatty Acids	A23187	A23187 plus PMA
	nmoles released/2 x 10^7 cells	
Oleic acid (18:1, n9)	1.98 ± 0.19	2.74 ± 0.12
Linoleic acid (18:21, n6)	0.56 ± 0.10	1.33 ± 0.10
Arachidonic acid (20:4, n6)	2.17 ± 0.35	6.96 ± 0.45

Human neutrophils (2 x 10^7 cells/ml) were stimulated with A23187 (3 μM) or A23187 plus PMA (50 nM) for 5 min at 37°C in the presence of BW755C, a dual inhibitor of cylooxygenase/lipoxygenase. Fatty acids released were analyzed as described in "Materials and Methods". Values represent the mean ± SE. (n=3).

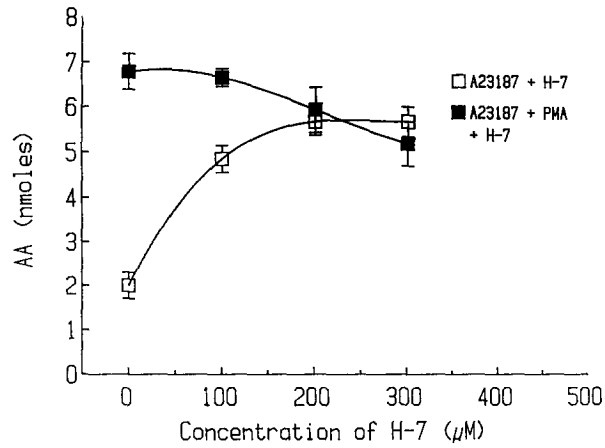

Figure 1. Effect of H-7 on the release of arachidonic acid: Neutrophils, preincubated with different concentrations of H-7 for 2 min, were stimulated with A23187 or A23187 plus PMA in the presence of BW755C as described in the legend to Table 1. Arachidonic acid released was quantitated as described in "Materials and Methods". Values represent the mean ± S.E. (n=3).

a 1.5-2.5 fold increase in the absolute amounts of released oleic, linoleic and arachidonic acids in the presence of H-7 (100-300 μM). These results suggest that H-7 may also activate a nonspecific PLA_2 similar to that observed in the presence of PMA, a putative activator of protein kinase C. It is rather surprising that both activator and inhibitor of PKC potentiate the release of all three unsaturated fatty acids presumably either by activating a nonspecific PLA_2 or by modulating the specificity of arachidonic acid-specific PLA_2. The mechanism(s) by which these two modulators affect the release of unsaturated fatty acids from the sn-2 position of neutrophil phosphoglycerides (PLA_2-like activity) in A23187-stimulated neutrophils remain unclear although the possibility of differential effects of protein kinase C isozymes can be implicated (24-28). The potentiating effect of H-7 on A23187-stimulated neutrophils does not appear to be due to a lytic effect on the

Table 2. Effect of H-7 on A23187-Stimulated Release of Oleic and Linoleic Acids in Neutrophils

	Concentration of H-7 (μM)			
Fatty Acid	0	100	200	300
Oleic acid (18:1, n9)	1.69 ± 0.10	2.32 ± 0.13	2.47 ± 0.11	2.31 ± 0.10
Linoleic acid (18:2., n6)	0.56 ± 0.10	1.01 ± 0.10	1.17 ± 0.10	0.96 ± 0.10

Released oleic and linoleic acids from stimulated neutrophils were analyzed as described in "Materials and Methods". Values represent the mean ± S.E. (n=3).

Table 3. Effect of H-7 on PMA-Primed and A23187-Stimulated Release of Oleic and Linoleic Acids in Neutrophils

	Concentration of H-7 (μM)			
Fatty Acid	0	100	200	300
Oleic acid (18:1, n9)	2.74 ± 0.12	3.19 ± 0.17	3.48 ± 0.44	2.38 ± 0.15
Linoleic acid (18:2., n6)	1.33 ± 0.10	1.49 ± 0.12	1.17 ± 0.15	0.97 ± 0.10

Released oleic and linoleic acids from PMA-primed and A23187-stimulated neutrophils were analyzed and quantitated as described in "Materials and Methods". Values represent the mean ± S.E. (n=3).

membrane as H-7 alone exerts no effect on the release of fatty acids from neutrophil phosphoglycerides.

In contrast, H-7 (300 μM) inhibited the PMA-mediated and synergistic release of oleic acid, linoleic acid and arachidonic acid by 35, 47 and 33%, respectively, in A23187-stimulated neutrophils (Fig. 1 and Table 3). Lesser concentrations of H-7 were also effective in attenuating the PMA-primed release of unsaturated fatty acids in A23187-stimulated neutrophils. These results are in partial agreement with previous reports in which H-7 has been known to inhibit a stimulus-induced release of arachidonic acid (6). Our results thus imply that inhibitory effects of H-7 on PMA-primed synergism in regards to the release of unsaturated fatty acids including arachidonic acid may be due to the inhibition of PKC activation, resulting in the presence of PMA.

In summary, we conclude that H-7 enhances the A23187-stimulated release of unsaturated fatty acids although it inhibits the PMA-primed release of these fatty acids. This differential effect of H-7 may likely be due to its differential influence on PLA_2 isoforms and it is also likely that the mechanism of action of PLA_2 activation in A23187-stimulated neutrophils differs from that involved in PMA-primed and A23187-stimulated neutrophils. These results suggest that one must exert great caution in the interpretation of the effect of H-7 in regards to the regulation of PLA_2 by a PKC-dependent mechanism despite its effect to attenuate PMA-primed increase in PLA_2-like activity.

Acknowledgments: This work was supported by a grant from the Heart and Stroke Foundation of Ontario. We would like to thank Miss Bev Healy for typing this manuscript.

REFERENCES

1. Nishizuka, Y. (1984) Nature **308**, 693-698
2. Wolfson, M., McPhail, L.C., Nasrallah, V.N., and Snyderman, R. (1985) J. Immunol. **135**, 2057-2062

3. Niedel, J.E. and Blackshear, P.J. (1986) In Phosphoinositides and Receptor Mechanisms (Putney Jr., J.W., ed.), Vol. 7, pp 47-88
4. Erusalimsky, J.D., Friedburg, I., and Rozengurt, E. (1988) J. Biol. Chem. **263**, 19188-19194
5. McIntyre, T.M., Reinhold, S.L., Prescott, S.M., and Zimmerman, G.A. (1987) J. Biol. Chem. **262**, 15370-15376
6. Parker, J., Daniel, L.W., and Waite, M. (1987) J. Biol. Chem. **262**, 5385-5393
7. Feinstein, M.B., and Halenda, S.P. (1988) Experientia **44**, 101-104
8. Halenda, S.P., Banga, H.S., Zavoico, G.B., Lau, L.-F., and Feinstein, M.B. (1989) Biochemistry **28**, 7356-7363
9. Hidaka, H., Inagaki, M., Kawamoto, S., and Sasaki, Y. (1984) Biochemisitry **23**, 5036-5041
10. Berkow, R.L., Dodson, R.W., and Kraft, A.S. (1987) J. Leukocyte Biol. **41**, 441-446
11. Fuse, I., and Tai, H.-H. (1988) Biochim. Biphys. Acta **972**, 54-59
12. Wright, C.D., and Hoffman, M.D. (1986) Biochem. Biophys. Res. Commun. **135**, 749-755
13. Wolf, M., and Baggiolini, M. (1988) Biochem. Biophys. Res. Commun. **154**, 273-1279
14. Hannun, Y.A., and Bell, R.M. (1987) Science **235**, 670-674
15. Birrell, G.B., Hedberg, K.K., Habliston, D.L., and Griffith, O.H. (1989) J. Cell Biol. **109**, 214
16. Mahadevappa, V.G., and Powell, W.S. (1989) J. Cellular Biochem. **40**, 341-352
17. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. **37**, 911-919
18. Walsh, C.E., Waite, B.M., Thomas, M.J., and DeChatelet, L.R. (1981) J. Biol. Chem. **256**, 7228-7234
19. Walsh, C.E., DeChatelet, L.R., Chilton, F.H., Wykle, R.L.,and Waite, M. (1983) Biochim. Biophys. Acta **750**, 32-40
20. Chilton, F.H., and Connell, T.R. (1988) J. Biol. Chem. **263**, 5260-5265
21. McColl, S.R., Hurst, N.P., and Cleland, L.G. (1986) Biochem. Biophys. Res. Commun. **141**, 399-404
22. Volpi, M., Molski, T.F.P., Naccacne, P.H., Feinstein, M.B., and Sha'afi, R.I. (1985) Biochem. Biophys. Res. Commun. **128**, 594-600
23. Balsinde, J., Diez, E., Schuller, A., and Mollinedo, F. (1988) J. Biol. Chem. **263**, 1929-1936
24. Coussens, L., Parker, P.J., Rhee, L., Yang-Feng, T.L., Chen, E., Waterfield, M.D., Francke, V., and Ullrich, A. (1986) Science **233**, 859-866
25. Kosaka, Y., Ogita, K., Ase, K., Nomura, H., Kikkawa, U., and Nishizuko, Y. (1988) Biochem. Biophys. Res. Commun.
26. Tsukuda, M., Asaoka, Y., Sekiguchi, K., Kikkawa, U., and Nishizuka, Y. (1988) Biochem. Biophys. Res. Commun. **155**, 1387-1395
27. Sekiguchi, K., Tsukuda, M., Ase, K., Kikkawa, U., and Nisihizuka, Y. (1987) J. Biochem. 759-765
28. Ohno, S., Kawasaki, H., Kono, Y., Inagak, M., Hidaka, H., and Suzuki, K. (1988) Biochemistry **27**, 2083-2087.