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DEMONSTRATION OF INOSITOL PHOSPHATE 5-PHOSPHOMONOESTERASE ACTIVITY IN RABBIT NEUTROPHILS: ABSENCE OF A ROLE FOR PROTEIN KINASE C

S.P. Kennedy, R.I. Sha'afi, and E.L. Becker

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

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SUMMARY: Rabbit peritoneal neutrophils, permeabilized with Triton X-100, contain inositol phosphate 5-phosphomonoesterase activity capable of converting [^{3}H]inositol 1,4,5-trisphosphate (Ins-1,4,5-P_3) to [^{3}H]inositol 1,4-bisphosphate. This activity is found predominantly associated with the soluble component of fractionated neutrophils. It is comprised of specific and non-specific activities toward Ins-1,4,5-P_3 which can be separated by cation exchange chromatography. Treatment of neutrophils with phorbol 12-myristate 13-acetate (PMA) prior to permeabilization does not affect the rate of Ins-1,4,5-P_3 breakdown by these cells. In addition, activation of endogenous protein kinase C in a soluble fraction prepared from neutrophils does not affect the specific inositol phosphate 5-phosphomonoesterase activity of this fraction. Taken together, these results provide evidence that activation of protein kinase C in the neutrophil does not affect its 5-phosphomonoesterase activity. Unlike platelets, the phosphorylation of a 5-phosphomonoesterase, if it occurs, may not play a role in the inhibitory effects of PMA on neutrophil responsiveness.

Exposure of neutrophils to chemoattractants causes the phosphodiesteratic cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C (1-3). The products of this hydrolysis, 1,2-diacylglycerol (DG) and inositol 1,4,5-trisphosphate (Ins-1,4,5-P_3), activate protein kinase C and mobilize Ca^{2+} from the endoplasmic reticulum, respectively (1-4). Both processes are thought to be involved in the initiation of neutrophil responses to chemoattractants such as superoxide generation and degranulation (5). However, results obtained in studies using phorbol esters, which bind to and activate

ABBREVIATIONS
Ins-1,4,5-P_3, inositol 1,4,5-trisphosphate; Ins-1,4-P_2, inositol 1,4-bisphosphate; Ins-P, inositol monophosphate; PMA, phorbol 12-myristate 13-acetate; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Mes, 2-(N-morpholino) ethanesulfonic acid; EGTA, [ethylenbis (oxyethylenenitrilio)] tetraacetic acid: H-7, 1-(5-isoquinoline-sulfonyl)2-methyl piperazine.

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protein kinase C, have suggested that the protein kinase C pathway may also function as an internal feedback system designed to modulate calcium signaling (6-11). Specifically, phorbol ester pretreatment of neutrophils leads to inhibition of fMet-Leu-Phe induced Ca^{2+} mobilization, and this inhibition is distal to the binding of fMet-Leu-Phe to its receptor (8). Recent data suggest that the activation of protein kinase C may block the ability of a guanine nucleotide regulatory protein (G protein), which is linked to the chemoattractant receptor, to activate phospholipase C (12,13). This may occur through direct phosphorylation of one or more components of the G protein (14).

Alternatively, activation of protein kinase C and inhibition of Ca^{2+} mobilization may result from an increased hydrolysis of Ins-1,4,5-P_3 to inositol 1,4-bisphosphate (Ins-1,4-P_2), which is unable to stimulate Ca^{2+} mobilization (15). It has recently been reported that a purified soluble platelet inositol phosphate 5-phosphomonoesterase can be phosphorylated and activated in vitro by protein kinase C (15). The phosphorylated 5-phosphomonoesterase is thought to be the 47 kDa (P47) protein kinase C substrate (see however, 16) originally associated with platelet degranulation (15,17). Furthermore, permeabilized platelets pretreated with protein kinase C activators display an increased conversion of Ins-1,4,5-P_3 to Ins-1,4-P_2 (18).

The present studies were undertaken to test for the presence of inositol phosphate 5-phosphomonoesterase activity and to examine the effect of protein kinase C activation on this activity in rabbit peritoneal neutrophils.

MATERIALS AND METHODS

In experiments using permeabilized cells, rabbit neutrophils were collected from a 4 h peritoneal exudate elicited with 0.1% oyster glycogen in sterile saline, washed twice and resuspended in 135 mM NaCl, 5 mM KCl, 5 mM glucose, and 10 mM Hepes, pH 7.3 (19). Inositol phosphate 5-phosphomonoesterase activity of these cells was determined by incubating 20 µl of a cell suspension (5×10^5 cells) with 0.05% Triton X-100, 1 mM EGTA, 3 mM $MgCl_2$ and 15 µM [^{3}H]Ins-1,4,5-P_3, in a total volume of 50 µl at 25°C. Reactions were stopped by the addition of 3 ml H_2O (90°C). Samples were cooled and then applied to columns containing 1 ml Dowex anion exchanger, and [^{3}H]inositol phosphates were separated as previously described (20).

Neutrophil cytosol (120,000 x g supernatant) was obtained by homogenization of neutrophils in a sucrose buffer followed by differential centrifugation

as described (21). Cytosol protein concentrations ranged from 10-12 mg/ml in the experiments described. Cytosolic 5-phosphomonoesterase activity was assayed by incubating cytosol (50 µg) with 3 mM $MgCl_2$, 15 µM [^{3}H]Ins-1,4,5-P_3, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 50 mM Hepes, pH 7.3 in 50 µl at 37°C for various times as indicated in the text. Reactions were stopped and [^{3}H]inositol phosphates analyzed as above. Protein was determined by the method of Lowry (22).

To assay for endogenous regulation of cytosolic 5-phosphomonoesterase activity, 1 ml freshly prepared cytosol was incubated with the various compounds indicated in the text in a total volume of 2 ml at 37°C for 20 min. Reactions were stopped by the addition of 3 ml cold cation exchange buffer consisting of 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 20 mM Mes, pH 6. Samples were applied to individual 1 ml CM-52 columns equilibrated in buffer at 4°C. The columns were washed with 5 ml buffer to remove nonspecific 5-phosphomonoesterase activity and specific enzyme activity was eluted with 4 ml buffer containing 150 mM NaCl. A 25 µl aliquot of each fraction was assayed for activity for 10 min at 37°C as described above. This procedure resulted in >85% recovery of total cytosolic activity towards Ins-1,4,5-P_3.

[^{3}H]Inositol 1,4,5-trisphosphate (4.0 Ci/mmol) and [^{3}H]inositol 1,4-bisphosphate (2.0 Ci/mmol) were purchased from New England Nuclear. Nonradioactive inositol phosphates were from Sigma. Phorbol 12-myristate 13-acetate was from Calbiochem. CM-52 was from Whatman and Dowex AG 1-X8 (formate form) was obtained from Bio-Rad. 1-(5-isoquinoline-sulfonyl)-2-methyl piperazine (H-7) was from Seikagaku America, Inc. (St. Petersburg, FL). All other reagents were reagent grade.

RESULTS AND DISCUSSION

Fig. 1 shows a time course of the conversion of [^{3}H]Ins-1,4,5-P_3 to [^{3}H]Ins-1,4-P_2, [^{3}H]Ins-P and [^{3}H]inositol by rabbit neutrophils permeabilized

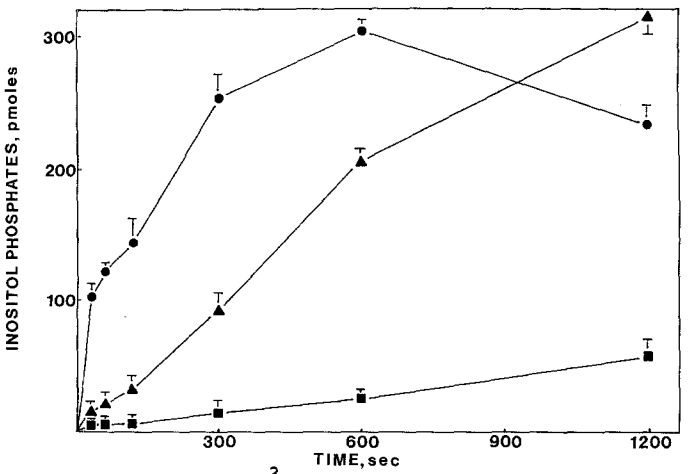

Figure 1 Dephosphorylation of [^{3}H]Ins-1,4,5-P_3 by permeabilized rabbit neutrophils. Neutrophils (5 x 10^5 cells) were incubated with 15 µM [^{3}H]Ins-1,4,5-P_3 and 0.05% Triton X-100 at 25°C for the indicated times. Reactions were stopped and samples analyzed for [^{3}H]Ins-1,4-P_2 (filled circles), [^{3}H]Ins-P (filled triangles) and [^{3}H]inositol (filled squares) as described in "Materials and Methods". The data shown are representative of two similar experiments performed using neutrophils from different rabbits.

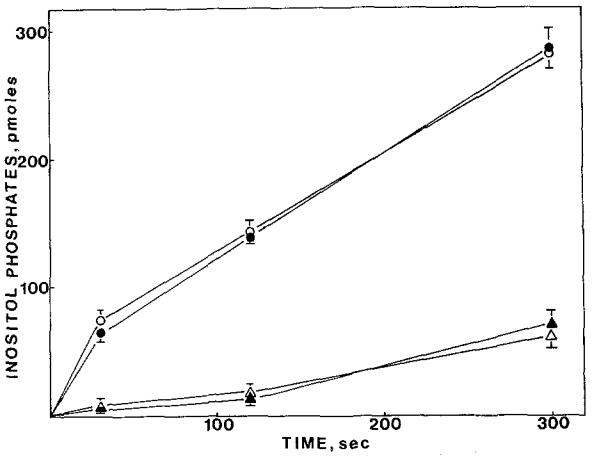

Figure 2 Effect of PMA pretreatment of permeabilized rabbit neutrophils on the dephosphorylation of [^{3}H]Ins-1,4,5-P_3. Neutrophils were preincubated with (closed symbols) or without (open symbols) 0.1 µg/ml PMA for 5 min at 25°C and aliquots (5 x 10^5 cells) were incubated with 15 µM [^{3}H]Ins-1,4,5-P_3 and Triton X-100 as described in Fig. 1. Samples were analyzed for [^{3}H]Ins-1,4-P_2 (circles) and [^{3}H]Ins-P (triangles). The data shown are representative of two similar experiments performed using neutrophils from different rabbits.

with 0.05% Triton X-100. The dephosphorylation of Ins-1,4,5-P_3 occurred rapidly [1.0 ± 0.1 nmol/min/10^7 cells (mean ± S.E.M., n=3)], with a hydrolysis pattern indicating a stepwise degradation of Ins-1,4,5-P_3 to Ins-1,4-P and Ins-P similar to that reported for platelets (18).

In order to examine the effect of protein kinase C activation on Ins-1,4,5-P_3 dephosphorylation, neutrophils were preincubated with PMA before permeabilization. As shown in Fig. 2, pretreatment of neutrophils for 5 min with 0.1 µg/ml PMA clearly does not affect the breakdown of Ins-1,4,5-P_3 to Ins-1,4-P_2 and Ins-P. Similar results were obtained in experiments where neutrophils were pretreated (0.5 - 20 min) with 1 µg/ml PMA, 5 µg/ml oleoylacetyl glycerol or 25 µM H-7 (data not shown). These data indicate that protein kinase C activation in intact neutrophils does not affect their total hydrolytic activity towards Ins-1,4,5-P_3, unlike platelets in which phorbol ester pretreatment resulted in an enhanced breakdown of Ins-1,4,5-P_3 and Ins-1,4,-P_2 (18). We surmised that the neutrophil activity was most likely comprised of specific and nonspecific activities since phosphatase substrates such as p-nitrophenyl phosphate, glucose-6-phosphate and β-glycero-

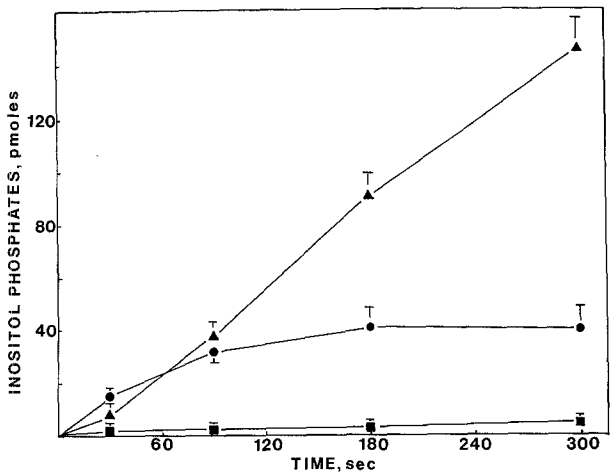

Figure 3 Dephosphorylation of [^{3}H]Ins-1,4,5-P_3 by cytosol prepared from rabbit neutrophils. Cytosol (50 µg) was incubated with 15 µM[^{3}H]Ins-1,4,5-P_3 for the indicated times at 37°C. Reactions were stopped and analyzed for [^{3}H]Ins-1,4-P_2 (filled circles), [^{3}H]Ins-P (filled triangles) and [^{3}H]inositol (filled squares) as described in "Materials and Methods". Values shown are average of duplicate determinations and the figure shown is representative of three similar experiments.

phosphate inhibit some of the breakdown of Ins-1,4,5-P_3 to Ins-1,4-P_2, and since solubilization with Triton X-100 exposes Ins-1,4,5-P_3 to all cellular compartments (data not shown). Neutrophils were fractionated, therefore, and a cytosol fraction prepared as described under "Materials and Methods", in order to examine the effect of endogenous protein kinase C activation on soluble 5-phosphomonoesterase activity.

The cytosol fraction contained 70 ± 5% (mean ± S.E.M., n=3) of the total unfractionated activity towards Ins-1,4,5-P_3. As shown in Fig. 3, cytosol converted [^{3}H]Ins-1,4,5-P_3 to [^{3}H]Ins-1,4-P_2, [^{3}H]Ins-P and [^{3}H]inositol, however, the time course and pattern of Ins-1,4-P_2 hydrolysis was different from that observed in permeabilized cells. In fact, Ins-1,4-P_2 was hydrolyzed rapidly, at a 5-fold higher rate than Ins-1,4,5-P_3 when assayed separately using equal concentrations (15 µM) of each radiolabeled inositol phosphate. This result contrasts with that reported for platelet cytosol which did not exhibit any hydrolytic activity towards Ins-1,4-P_2 (23). The breakdown of Ins-1,4,5-P_3 by this neutrophil soluble fraction was partially inhibited by p-nitrophenyl phosphate, suggesting that it also contained nonspecific activity towards Ins-1,4,5,-P_3 (data not shown). In order to examine only

TABLE I

Separation of Specific and Nonspecific Hydrolytic Activities Towards Ins-1,4,5-P_3 by CM-52 Chromatography

CM-52 fraction	[^{3}H]Ins-1,4,5-P_3 hydrolysis product*	
	Ins-1,4-P_2	Ins-P
Nonspecific	30	106
Specific	152	ND

* Total cytosolic activity towards Ins-1,4,5-P_3 was separated into specific and nonspecific fractions based on the ability of the enzyme activity to bind or not bind, respectively, to a 1 ml CM-52 column as described in "Materials and Methods". Total volumes of the specific and nonspecific fractions were 4 and 10 ml, respectively. Results are expressed as pmoles [^{3}H]Ins-1,4-P_2 and [^{3}H]Ins-P produced after incubation of a 25 µl aliquot of each fraction with 15 µM [^{3}H]Ins-1,4,5-P_3 for 10 min at 37°C and are representative of five identical experiments performed. ND - none detected.

specific 5-phosphomonoesterase activity it was necessary to remove nonspecific activities towards Ins-1,4,5-P_3 and Ins-1,4,-P_2. As shown in Table I, the nonspecific activity, which comprised about 50% (n=5) of the total activity and did not bind to CM-52, hydrolyzed Ins-1,4,5-P_3 to both Ins-1,4-P_2 (22%) and Ins-P (78%). Conversely, the specific enzyme bound to CM-52 and could be eluted with a buffer containing 150 mM NaCl. This enzyme fraction hydrolyzed Ins-1,4,5-P_3 only to Ins-1,4-P_2, even after prolonged incubation with substrate.

As shown in Table II, cytosol was preincubated with or without activators of protein kinase C and the effect on specific 5-phosphomonoesterase activity was examined after separation from nonspecific enzyme on CM-52. It must be pointed out that this cytosol contains protein kinase C activity as measured by histone phosphorylation (the specific activity was 200 pmol ^{32}P incorporated in histone III S per min. per mg protein at 25°C). In the three experiments performed, activation of endogenous protein kinase C by the addition of 100 µg/ml phosphatidylserine, 0.1 µg/ml PMA, and 200 µM Ca^{2+} (PKC Activation Mixture), in the presence or absence of 50 µM H-7, did not significantly affect the specific 5-phosphomonoesterase activity (Table II). The pretreatments also did not affect the nonspecific enzyme activity (data not shown). These

TABLE II

Effect of Activation of Endogenous Cytosolic Protein Kinase C on Cytosolic Inositol Phosphate 5-Phosphomonoesterase Activity

Condition*	Activity (pmol/min)		
	Exp. 1	Exp. 2	Exp. 3
Control	11.0 ± 0.8	19.8 ± 0.7	15.1 ± 0.1
Mg^{2+}, ATP	10.2 ± 0.2	18.9 ± 0.9	16.8 ± 0.2
PKC Activation Mixture	10.8 ± 0.1	19.7 ± 0.2	15.3 ± 0.2
PKC Activation Mixture, Mg^{2+}, ATP	10.9 ± 0.5	19.6 ± 0.5	16.5 ± 0.4
PKC Activation Mixture, Mg^{2+}, ATP, H-7	-	-	17.1 ± 0.1

* Cytosol (1 ml, 10-12 mg) was incubated with the indicated combination of buffer (control), 5 mM $MgCl_2$, 20 µM ATP, 50 µM H-7, or the PKC activation mixture (100 µg/ml phosphatidylserine, 0.1 µg/ml PMA, 200 µM free Ca^{2+}) for 20 minutes at 37°C in a total volume of 2 ml. Three ml of cold cation exchange buffer was added to stop the reaction and specific enzyme was isolated and activity assayed as described in "Materials and Methods". Each experiment was performed with a separate cytosol preparation. Values are the mean ± S.E.M. of duplicate determination.

results indicate that activation of endogenous protein kinase C does not affect the soluble specific 5-phosphomonoesterase activity of neutrophils. Purification of the 5-phosphomonoesterase is required to determine if it is phosphorylated by protein kinase C.

In summary, the precise site at which PMA interrupts the excitation response coupling sequence and inhibits the stimulated rise in the intracellular concentration of calcium in the neutrophil remains to be defined. The present report suggests that, unlike in platelets, the neutrophil soluble inositol phosphate 5-phosphomonoesterase is not a potential site and may not play a role in the inhibitory effect of PMA.

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REFERENCES

1. Bradford, P.G. and Rubin, R.P. (1986) J. Biol. Chem. 261, 15644-15647.
2. Volpi, M., Yassin, R., Naccache, P.H. and Sha'afi, R.I. (1983) Biochem. Biophys. Res. Commun. 112, 957-964.

3. Snyderman, R., Smith, C.D. and Verghese, M.W. (1986) J. Leuk. Biol. 40, 785-800.
4. Berridge, M.J. (1987) Ann. Rev. Biochem. 56, 159-193.
5. Snyderman, R. and Pike, M.C. (1984) Ann. Rev. Immunol. 2, 257-281.
6. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847-7851.
7. Nishizuka, Y. (1986) Science 233, 305-312.
8. Naccache, P.H., Molski, T.F.P., Borgeat, P., White, J.R. and Sha'afi, R.I. (1985) J. Biol. Chem. 260, 2125-2131.
9. Rittenhouse, S.E. and Sasson, J.P. (1985) J. Biol. Chem. 260, 8657-8660.
10. Zavoico, G.B., Halenda, S.P., Sha'afi, R.I. and Feinstein, M.B. (1985) Proc. Natl. Acad. Sci. 82, 3859-3862.
11. Orellana, S.A., Solski, P.A. and Brown, J.H. (1985) J. Biol. Chem. 260, 5236-5239.
12. Smith, C.D., Uhing, R.J. and Snyderman, R. (1987) J. Biol. Chem. 262, 6121-6127.
13. Matsumoto, T., Molski, T.F.P., Kanaho, Y., Becker, E.L. and Sha'afi, R.I. (1987) Biochem. Biophys. Res. Commun. 143, 489-498.
14. Katada, T., Gilman, A.G., Watanabe, Y., Bauer, S. and Jakobs, K.H. (1985) Eur. J. Biochem. 151, 431-437.
15. Connolly, T.M., Lawing, W.J. and Majerus, P.W. (1986) Cell 46, 951-958.
16. Tyers, M., Rachubinski, R.A., Steward, M.I., Varrichio, A.M., Shorr, R.G.L., Haslam, R.J. and Harley, C.B. (1988) Nature 333, 470-473.
17. Haslam, R.J. and Lynham, J.A. (1977) Biochem. Biophys. Res. Commun. 77, 714-722.
18. Molina y. Vedia, L.M. and Lapetina, E.G. (1986) J. Biol. Chem. 261, 10493-10495.
19. Showell, H.J., Williams, D., Becker, E.L., Naccache, P.H. and Sha'afi, R.I. (1979) J. Reticuloendothel. Soc. 25, 139-150.
20. Downes, C.P. and Michell, R.H. (1981) Biochem. J. 198, 133-140.
21. Huang, C.-K., Devanney, J.F. and Kanaho, Y. (1987) Biochem. Biophys. Res. Commun. 142, 242-250.
22. Lowry, O.H., Rosebrough, N.T., Farr, A.L. and Randall, R.T. (1951) J. Biol. Chem. 193, 265-275.
23. Connolly, T.M., Bross, T.E. and Majerus, P.W. (1985) J. Biol. Chem. 260, 7868-7874.
24. Sha'afi, R.I., Molski, T.F.P., Huang, C.-K. and Naccache, P.H. (1986) Biochem. Biophys. Res. Commun. 137, 50-60.
25. Sklar, L.A., Hyslop, P.A., Oades, Z.G., Omann, G.M., Jesaitis, A.J., Painter, R.G. and Cochrane, C.G. (1985) J. Biol. Chem. 260, 11461-11467.