Biochemical Pharmacology, Vol. 34, No. 11, pp. 1931–1935, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

ROLE OF ENHANCED INOSITOL PHOSPHOLIPID METABOLISM IN NEUTROPHIL ACTIVATION

TADAOMI TAKENAWA,* J. ISHITOYA, Y. HOMMA, M. KATO and Y. NAGAI
Department of Biochemistry, School of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

(*Received* 17 *July* 1984; *accepted* 1 *October* 1984)

Abstract—When guinea pig neutrophils were stimulated with chemotactic peptide [formylmethionyl-leucyl-phenylalanine (fMLP)], a marked release of lysosomal enzyme and production of superoxide anion were detected. The breakdown of phosphatidylinositol 4,5-bisphosphate (TPI) and the subsequent formation of diacylglycerol, phosphatidic acid and free arachidonic acid also occurred during the processes. Ca^{2+} ionophore A23187 caused an evident secretion of lysosomal enzyme but no superoxide anion production. Ca^{2+} ionophore also caused TPI breakdown to diacylglycerol although this breakdown was not as significant as that detected by fMLP. The tumor promotor tetradecanoylphorbol acetate (TPA), which is a strong activator of superoxide anion production but not a good stimulator of lysosomal enzyme secretion, did not cause a significant decrease of TPI or arachidonic acid release. Since TPA is known not to increase the intracellular Ca^{2+} level, these results suggest that lysosomal enzyme secretion is correlated closely with enhanced inositol phospholipid metabolism and Ca^{2+}-dependent processes. On the other hand, superoxide anion production seemed to be caused mainly by Ca^{2+}-independent processes, perhaps by protein kinase-C activation through newly formed diacylglycerol, when neutrophils were activated by chemotactic peptide.

Neutrophils are phagocytic cells whose primary function is to ingest and kill invading bacteria. The destruction of microorganisms was thought to be accomplished in part through superoxide anion production and lysosomal enzyme secretion. It has been generally considered that neutrophil activation occurs by raising the concentration of cytoplasmic free Ca^{2+}, which then triggers superoxide anion generation and lysosomal enzyme secretion [1–6]. However, the tumor promotor tetradecanoylphorbol acetate has been found recently to cause lysosomal enzyme secretion and superoxide anion generation without intracellular Ca^{2+} elevation [7]. Thus, it remains unclear by what mechanism superoxide anion generation and lysosomal enzyme secretion are induced.

We and other investigators [8–13] have demonstrated that "phosphatidylinositol (PI†) response" and arachidonic acid release are closely associated with neutrophil activation.

For this report, the effects of various activators on superoxide anion production, lysosomal enzyme secretion, and inositol phospholipid metabolism were studied, and the role of stimulated inositol phospholipid metabolism on superoxide anion production or lysosomal enzyme secretion is discussed.

* Author to whom all correspondence should be addressed.

† Abbreviations: PI, phosphatidylinositol; fMLP, formylmethionyl-leucyl-phenylalanine; TPI, phosphatidylinositol 4,5-bisphosphate; DPI, phosphatidylinositol 4-phosphate; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PAF, platelet activating factor; TPA, tetradecanoylphorbol acetate; acetyl DG, 1-oleoyl-2-acetyl glycerol; and HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

MATERIALS AND METHODS

[5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (95.4 Ci/mmole) and [^{32}P]orthophosphoric acid (carrier free) were obtained from New England Nuclear. Ca^{2+} ionophore A23187 was from the Calbiochem-Behring Co. The chemotaxin, formylmethionyl-leucyl-phenylalanine, cytochalasin B, *p*-nitrophenyl-acetyl-β-D-glucosamide, and tetradecanoylphorbol acetate were from the Sigma Chemical Co. 1-Alkyl-2-acetoyl-sn-glycero-3-phosphocholine (PAF) and arachidonic acid were from the Sedary Research Laboratories. 1-Oleoyl-2-acetylglycerol (acetyl DG) was a gift from the Nippon Shoji Co.

Guinea pig peritoneal neutrophils were obtained as previously described [8]. The cells were washed and resuspended ($1–5 \times 10^6$ cells/0.5 ml) in Eagle's minimum essential medium (EMEM) containing 10% guinea pig serum and 20 mM HEPES/NaOH buffer (pH 7.4). Superoxide anion production was measured as follows: Neutrophils (2×10^6 cells/0.5 ml) were stimulated by various agents in the presence of 80 μM cytochrome *c* with or without superoxide dismutase (10 μg/ml) for 10 min. The mixture was quickly chilled to 0° and then centrifuged at $500 \times g$ for 5 min. The supernatant fraction was transferred to 96 flat bottom wells for microtitration. The reduction of cytochrome *c* was monitored at 550 nm with a reference wave length of 540 nm. Labeling experiments with [^{32}P]P_i and [^{3}H]arachidonic acid were carried out as described previously [8,14]. For protein phosphorylation, neutrophils were incubated with 1 mCi of [^{32}P]P_i in phosphate-free EMEM containing 10% guinea pig serum and 20 mM HEPES/NaOH (pH 7.4) for 1 hr. The cells were washed twice and then resuspended in the fresh buffer. The cells (2×10^6 cells/0.5 ml) were stimulated with various agents for 3 min, followed

by the addition of chilled stop solution (100 mM NaF, 80 mM sucrose, 10 mM EDTA, 10 mM $NaHPO_4$ and 20 mM HEPES/NaOH buffer (pH 7.4). The cells were collected by centrifugation at 500 *g* for 5 min and solubilized by sodium dodecyl sulfate (SDS)-containing solution. Samples were layered on the top of slab gel (5–10% gradient acrylamide gel) and electrophoresized by the method of Laemmli [15]. Gels were fixed and autoradiographed using Kodak X-Omat films.

RESULTS

Effects of fMLP, Ca^{2+} ionophore A23187, TPA, PAF or acetyl DG on lysosomal enzyme secretion. As shown in Fig. 1A, fMLP and Ca^{2+} ionophore A23187 were found to be potent releasers of *N*-acetyl-β-D-glucosaminidase. Among them, Ca^{2+} ionophore was the strongest inducer. On the other hand, TPA, PAF and acetyl DG released the lysosomal enzyme only when neutrophils were treated with high concentrations of the agents.

Effects of fMLP, Ca^{2+} ionophore A23187, TPA, PAF or acetyl DG on superoxide anion production. TPA was found to be the strongest inducer of superoxide anion among the drugs examined (Fig. 1B). In this case, superoxide anion production was induced with TPA at concentrations 100–1000 times lower than those needed for releasing lysosomal enzyme. Chemotactic peptide, PAF and acetyl DG were found to be moderate inducers of superoxide anion. However, Ca^{2+} ionophore A23187 did not cause the marked production of superoxide anion. These results suggest that lysosomal enzyme secretion is primarily caused by the Ca^{2+}-dependent processes, and that superoxide anion production is mainly

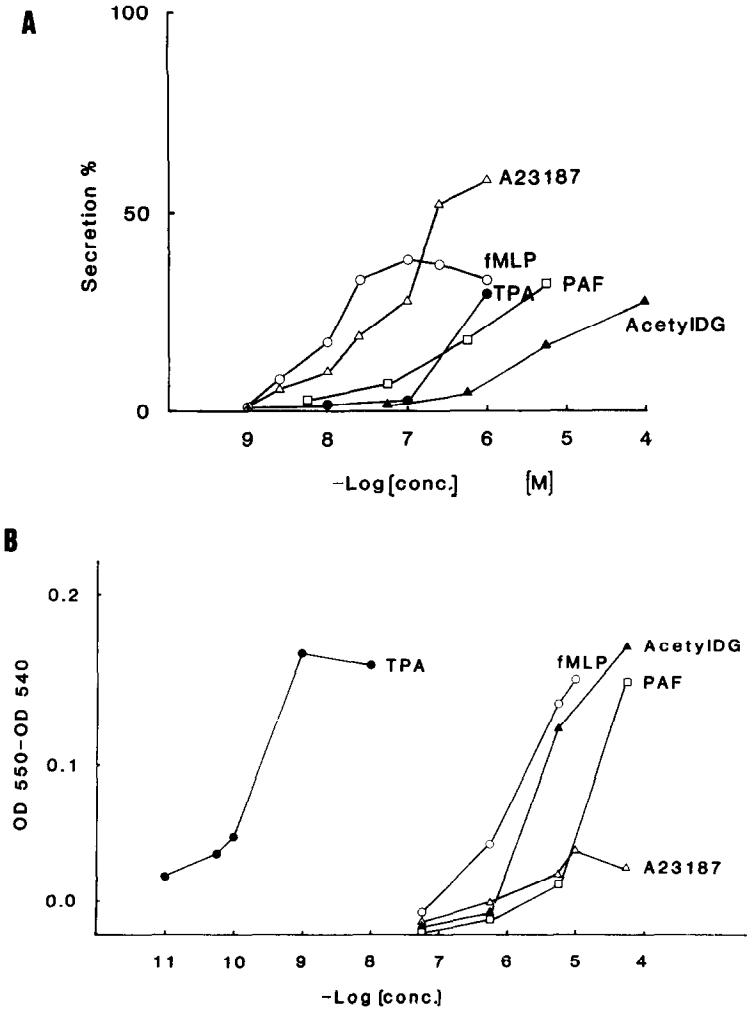

Fig. 1. Effects of fMLP, A23187, TPA, PAF and acetyl DG on lysosomal enzyme secretion (A) and superoxide anion production (B). (A). After neutrophils were treated with 5 μg/ml of cytochalasin B for 10 min at 37°, reaction was started by the addition of stimulants and was continued for 5 min. Aliquots of the supernatant fraction were used to measure secreted *N*-acetyl-β-D-glucosamidase. Abbreviations used are: TPA, tetradecanoylphorbol acetate; A23187, Ca^{2+} ionophore A23187; fMLP, formylmethionyl-leucyl-phenylalanine; PAF, platelet activating factor; and acetyl DG, 1-oleoyl-2-acetylglycerol. (B). Neutrophils were stimulated by various drugs for 10 min, and cytochrome *c* reduction was monitored at 550 nm with a reference of 540 nm.

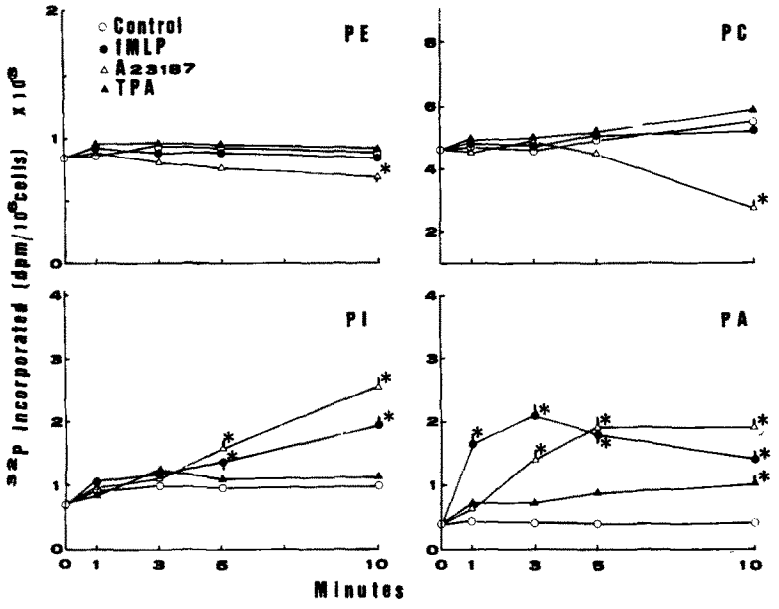

Fig. 2. Effects of fMLP, A23187 and TPA on $[^{32}P]P_i$ incorporation into various phospholipids. Neutrophils were incubated with 1 mCi of $[^{32}P]P_i$ in phosphate-free EMEM containing 10% guinea pig serum and 10 mM HEPES/NaOH (pH 7.4) buffer for 1 hr at 37°. After the cells were washed and resuspended in fresh medium, aliquots of the cells (2×10^6 cells) were stimulated by the addition of 1×10^{-6} M fMLP, 1×10^{-6} M A23187 or 10 ng/ml of TPA. Values are means ± S.E. of triplicate determinations. Asterisks indicate a significant difference from control ($P < 0.01$).

caused by Ca^{2+}-independent processes since TPA and acetyl DG do not cause an enhancement of Ca^{2+} mobilization [7,16].

Effects of fMLP, Ca^{2+} ionophore A23187 or TPA on $[^{32}P]P_i$ incorporation into various phospholipids. As shown in Fig. 2, fMLP (1 μM) and Ca^{2+} ionophore A23187 (1 μM) stimulated the incorporation of $[^{32}P]P_i$ into phosphatidylinositol (PI) and phosphatidic acid (PA). However, TPA did not enhance the incorporation into PI. In addition, the incorporation into PA was found to be very slightly stimulated by TPA. These results show that chemotactic peptide causes "PI response" but TPA does not.

Effects of fMLP, Ca^{2+} ionophore A23187 or TPA on the redistribution of $[^3H]$arachidonic acid-labeled lipids. Chemotactic peptide liberated arachidonic acid mainly from PI for the initial periods up to 3 min but, with 10 min of incubation, arachidonic acid release from phosphatidylcholine (PC) and phosphatidylethanolamine (PE) was evident (Fig. 3). Thus, it seems likely that the activation of PI-specific phospholipase C occurred first and was followed by the activation of phospholipase A_2. On the other hand, Ca^{2+} ionophore A23187 liberated $[^3H]$ arachidonic acid nonspecifically from PC, PE and PI in company with an increase of $[^3H]$arachidonic acid in the phosphatidic acid and free fatty acid fractions. In this case, it appeared that primarily phospholipase A_2 activation has occurred. But TPA did not release arachidonic acid with incubation for 10 min, though a slight increase of $[^3H]$arachidonic acid in phosphatidic acid fraction was observed. These results also show that the "PI response" is a phenomenon closely associated with Ca^{2+} mobilization.

Effects of fMLP, Ca^{2+} ionophore A23187 or TPA on ^{32}P-labeled TPI, DPI and PA. As shown in Fig.

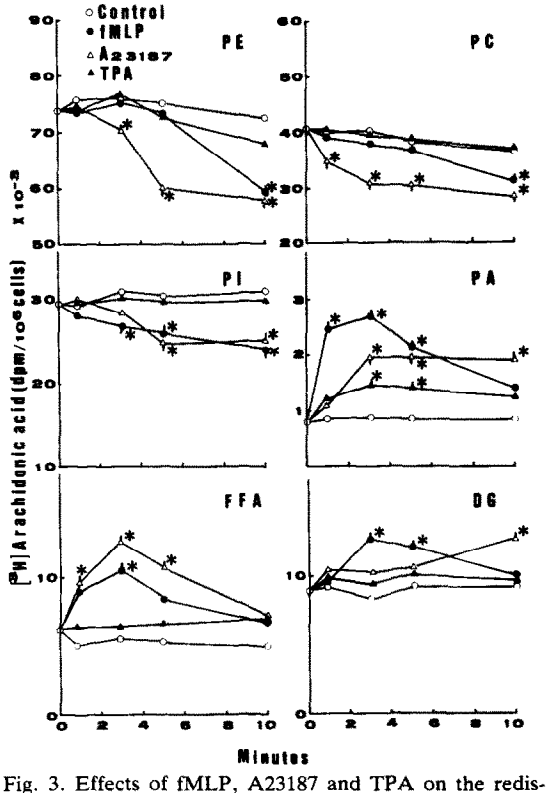

Fig. 3. Effects of fMLP, A23187 and TPA on the redistribution of $[^3H]$arachidonic acid moiety from $[^3H]$arachidonyl lipds. Neutrophils were incubated with $[^3H]$arachidonic acid for 1 hr at 37°. The cells were washed to remove free $[^3H]$arachidonic acid and stimulated with 1×10^{-6} M fMLP, 1×10^{-6} M A23187 or 10 ng/ml of TPA. Values are means ± S.E. of triplicate determinations. Asterisks indicate a significant difference from control ($P < 0.01$).

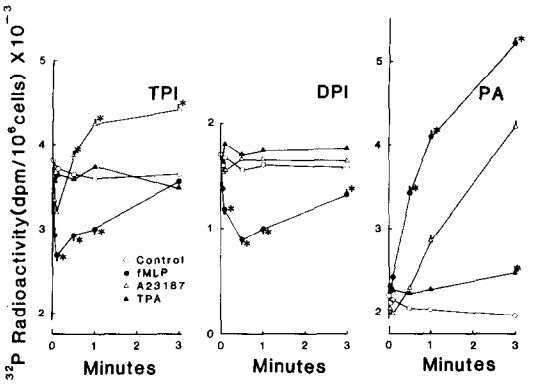

Fig. 4. Effects of fMLP, A23187 and TPA on the ^{32}P-labeling of TPI, DPI and PA. Neutrophils were incubated with [^{32}P]P_i for 1 hr at 37°. The cells were washed and stimulated with 1×10^{-6} M fMLP, 1×10^{-6} M A23187 or 10 ng/ml of TPA. Values are means ± S.E. of triplicate determinations. Asterisks indicate a significant difference from control ($P < 0.01$).

4, chemotactic peptide caused a very rapid decrease in [^{32}P]TPI. This decrease reached a maximum at 10 sec after stimulation, and then recovery synthesis of TPI became evident. Ca^{2+} ionophore also caused a decrease although it was fairly weak. TPA, however, did not cause the breakdown of TPI. Concerning DPI, chemotactic peptide also caused a decrease in [^{32}P]DPI and the maximum decrease was observed at 30 sec, followed by a compensatory resynthesis of DPI. TPA did not affect ^{32}P-labeling of DPI. PA labeling by ^{32}P increased with time without a transient decrease by chemotactic peptide and Ca^{2+} ionophore, as shown in Fig. 2. TPA did not increase the labeling significantly for the short periods.

Protein phosphorylation. The chemotactic peptide significantly increased the phosphorylation of 45K, 47K, 63K, 68K and 72K protein (Fig. 5). Other stimulants, such as Ca^{2+} ionophore, TPA and acetyl DG, also enhanced the [^{32}P]P_i incorporation into the same proteins. Among them, TPA and acetyl DG phosphorylated the proteins very strongly. Ca^{2+} ionophore A23187, which is a good releaser of lysosomal enzyme but not a good inducer of superoxide anion, stimulated the phosphorylation of the proteins weakly although the phosphorylation of 63K protein was, on the contrary, more marked than that by TPA. These differences may cause the different functions, lysosomal enzyme secretion and superoxide anion production.

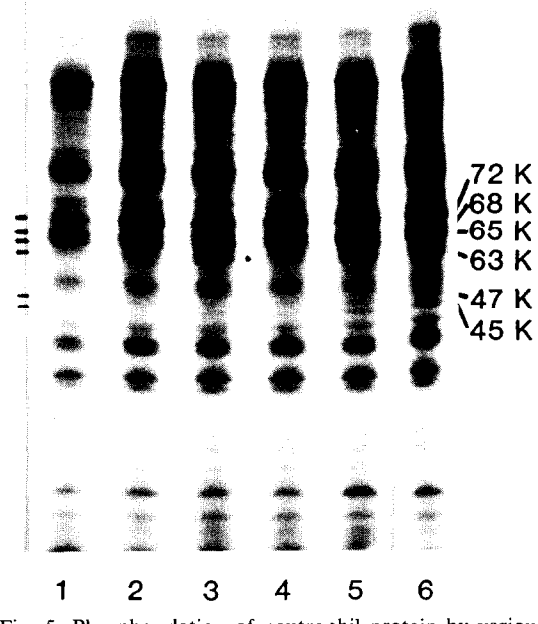

Fig. 5. Phosphorylation of neutrophil protein by various stimulants. Neutrophils were incubated with [^{32}P]P_i in phosphate-free EMEM containing 10% guinea pig serum and 10 mM HEPES/NaOH (pH 7.4) buffer for 1 hr at 37°. After the cells were washed and resuspended in fresh medium, neutrophils were stimulated by various drugs for 3 min, and the phosphorylated proteins were analyzed by SDS- polyacrylamide gel electrophoresis (5–15% gel) and autoradiography. Key: (1) control, (2) fMLP (1 μM), (3) A23187 (1 μM), (4) PAF (1 μM), (5) TPA (10 ng/ml), and (6) acetyl DG (10 μg/ml).

DISCUSSION

The chemotactic peptide, fMLP, caused both lysosomal enzyme secretion and superoxide anion to function effectively. fMLP stimulated TPI breakdown to diacylglycerol and inositol triphosphate very rapidly, as shown in Fig. 3. DG fraction in this experiment may have been contaminated with monohydroxyicosatetraenoic acid metabolites. But, when DG was separated by two-dimensional thin-layer chromatography according to Walsh *et al.* [9], similar results were obtained. Therefore, it was thought that most radioactivity was derived from DG by itself. Since diacylglycerol is known to be an activator of protein kinase-C [17,18], protein kinase-C activation may have taken place. It has been reported that TPI breakdown is closely related to Ca^{2+} mobilization, which might be initiated by another product of TPI breakdown, inositol triphosphate, as suggested by Streb and other investigators [19–21]. The resultant increase in intracellular free Ca^{2+} and DG may cause the activation of calmodulin-dependent kinase and Ca^{2+}-dependent processes. Thus, chemotactic peptide could cause the activation of both kinases, protein kinase-C and calmodulin-dependent kinase.

On the other hand, TPA and acetyl DG, which are known as protein kinase-C activator [17,18,22], did not affect the metabolism of inositol phospholipids markedly. Since it was known that these agents do not increase the intracellular Ca^{2+} [16], protein kinase-C activation rather than calmodulin-dependent kinase might be induced. In this case, participation of arachidonic acid metabolites could be ruled out, since TPA and acetyl DG did not cause arachidonic acid release. These agents activated superoxide anion production more strongly than lysosomal enzyme secretion, although high concentrations of the agents were found to induce both functions. On the contrary, Ca^{2+} ionophore caused a significant release of lysosomal enzymes but not production of superoxide anion (Fig. 1). This agent also stimulated the accumulation of DG; but superoxide anion production was not induced significantly

by Ca^{2+} ionophore. Thus, it is not clear whether the amount of formed diacylglycerol was enough to activate protein kinase-C or another factor was necessary to activate protein kinase-C effectively.

Several experiments already have been done on the study of protein phosphorylation [23–25], though little is known about the role of phosphorylation in neutrophil functions. Recently, a close relationship between lysosomal enzyme secretion and the phosphorylation of 50K protein was reported [25]. But the role of Ca^{2+} on phosphorylation and neutrophil functions is still unclear. In this experiment, the phosphorylation pattern by Ca^{2+} ionophore stimulation was not much different from that by TPA although Ca^{2+} ionophore-stimulation made a fairly weak phosphorylation pattern compared to TPA stimulation and the 63K protein appeared to be more strongly phosphorylated by Ca^{2+} ionophore than by TPA. Now, it remains to be solved if these differences reflect the different functions, lysosomal enzyme and superoxide anion production.

REFERENCES

1. D. Romeo, G. Zabucch, N. Miani and F. Rossi, *Nature, Lond.* **253**, 542 (1975).
2. J. E. Smolen, H. M. Korchak and G. Weissmann, *Biochim. biophys. Acta* **677**, 512 (1981).
3. J. R. White, P. H. Nacche, T. F. P. Molski, P. Bargeat and R. I. Sha'afi, *Biochem. biophys. Res. Commun.* **113**, 44 (1983).
4. R. I. Sha'afi, T. F. P. Molski, P. Borgeat and P. H. Naccache, *Biochem. biophys. Res. Commun.* **103**, 766 (1983).
5. H. J. Choen and M. E. Chovaniec, *J. clin. Invest.* **61**, 1088 (1978).
6. E. L. Becker, M. Sigman and J. M. Oliver, *Am. J. Path.* **95**, 81 (1983).
7. R. I. Sha'afi, J. R. White, T. F. P. Molski, J. Shefcyk, M. Volpi, P. H. Naccache and M. B. Feinstein, *Biochem. biophys. Res. Commun.* **114**, 638 (1983).
8. T. Takenawa, Y. Homma and Y. Nagai, *J. Immun.* **130**, 2849 (1983).
9. C. E. Walsh, B. M. Waite, M. J. Thomas and L. R. DeChatelet, *J. biol. Chem.* **256**, 7228 (1981).
10. P. H. Naccache, H. J. Showell, E. L. Becker and R. I. Sha'afi, *Biochem. biophys. Res. Commun.* **87**, 292 (1981).
11. C. N. Serhan, M. J. Broekman, H. M. Korchak, J. E. Smolen, A. J. Marcus and G. Weissmann, *Biochim. biophys. Acta* **762**, 420 (1983).
12. K. Yano, S. Nakajima and Y. Nozawa, *Fedn Eur. Biochem. Soc. Lett.* **161**, 296 (1983).
13. M. Volpi, R. Yassin, P. H. Naccache and R. I. Sha'afi, *Biochem. biophys. Res. Commun.* **112**, 957 (1983).
14. Y. Homma, K. Onozaki, T. Hashimoto, Y. Nagai and T. Takenawa, *J. Immun.* **129**, 1619 (1982).
15. U. K. Laemmli, *Nature Lond.* **227**, 680 (1970).
16. T. J. Rink, A. Sanchez and T. J. Hallam, *Nature, Lond.* **305**, 317 (1983).
17. K. Kaibuchi, K. Sano, M. Hoshijima, Y. Takai and Y. Nishizuka, *Cell Calcium* **3**, 323 (1982).
18. K. Sano, Y. Takai, J. Yamanishi and Y. Nishizuka, *J. biol. Chem.* **258**, 2010 (1983).
19. H. Streb, R. F. Irvine, H. J. Berridge and I. Schulz *Nature, Lond.* **306**, 67 (1983).
20. G. M. Burgess, P. P. Godfrey, J. S. McKinney, M. J. Berridge, R. F. Irvine and J. W. Putney, *Nature, Lond.* **309**, 63 (1984).
21. S. K. Joseph, A. P. Thomas, D. J. William, R. F. Irvine and J. P. Williamson, *J. biol. Chem.* **259**, 3077 (1984).
22. U. Kikkawa, Y. Takai, Y. Tanaka, R. Miyake and Y. Nishizuka, *J. biol. Chem.* **258**, 11442 (1983).
23. C. Schneider, M. Zanetti and D. Romeo, *Fedn Eur. Biochem. Soc.* **127**, 4 (1981).
24. P. C. Andrews and B. M. Babior, *Blood* **61**, 333 (1983).
25. J. R. White, C-K. Huang, J. M. Hill, P. H. Naccache, E. L. Becker and R. J. Sha'afi, *J. biol. Chem.* **259**, 8605 (1984).