Biochemical Pharmacology, Vol. 41, No. 10, pp. 1449–1454, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

THE PROTEIN KINASE C INHIBITOR, K252a, INHIBITS SUPEROXIDE PRODUCTION IN HUMAN NEUTROPHILS ACTIVATED BY BOTH PIP_2-DEPENDENT AND -INDEPENDENT MECHANISMS

BREDA M. TWOMEY, KARINA CLAY and M. MAUREEN DALE*
Department of Pharmacology, University College London, Gower Street, London WC1E 6BT, U.K.

(*Received* 31 *August* 1990; *accepted* 6 *December* 1990)

Abstract—We report that the putative protein kinase C inhibitor, K252a, at concentrations of 0.2 and 1 μM, inhibited the respiratory burst induced by fluoride and formyl-methionyl-leucyl-phenyl-alanine respectively, both in human neutrophils primed with a subthreshold dose of phorbol myristate acetate and in non-primed neutrophils. In addition, K252a effectively inhibited ConA-zymosan-mediated superoxide generation in Ca^{2+}-depleted neutrophils, with virtually maximal inhibition seen at 1 μM. These results suggest that protein kinase C is involved in the putative phosphatidylinositol bisphosphate-independent signal transduction mechanism of the respiratory burst as well as the pathway dependent on phosphatidylinositol bisphosphate hydrolysis.

Superoxide (O_2^-)† generation by human neutrophils occurs in response to a wide range of stimuli and it is believed to be an important factor in tissue damage [1]. The transduction pathways linking signal to NADPH oxidase activation have yet to be fully elucidated, but in many cells the link is believed to involve phosphatidylinositol bisphosphate (PIP_2) hydrolysis, with the liberation of inositol triphosphate (IP_3) which increases $[Ca^{2+}]_i$, and diacylglycerol (DAG) which activates protein kinase C (PKC). IP_3 and DAG are reported to act synergistically in several cell types [2, 3], and such synergism has also been reported for the neutrophil [4–6].

Recently two novel activation mechanisms for the neutrophil respiratory burst have been described, both being reported as PIP_2-independent, and one being proposed as PKC-independent as well [7–10].

To assess the role of PKC in these situations we have employed the use of a potent, PKC inhibitor, K252a (Fig. 1).

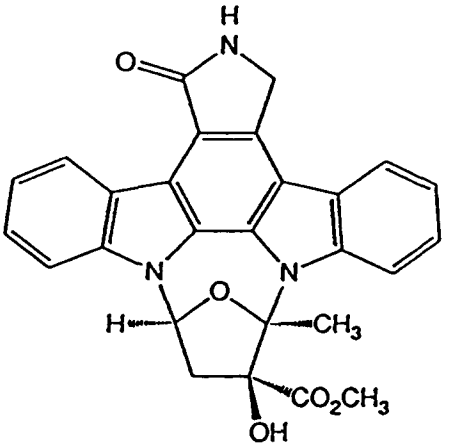

Fig. 1. The structure of K252a.

This compound has been reported to inhibit rat brain PKC with K_i values of 0.025 μM [11] and 0.47 μM [12], and human neutrophil PKC with K_i values of 0.27 μM [13] and 0.58 μM [14]. Although K252a has been described as a potent PKC inhibitor, it is not selective for PKC; it also inhibits other protein kinases such as cyclic AMP-dependent protein kinase (PKA) with K_i values of 0.018 μM [11], 0.20 μM [12] and 0.16 μM [13]. K252a has a K_i value of 0.02 μM for cyclic GMP-dependent protein kinase [11] and of 0.30 μM [12] for Ca^{2+}/calmodulin-dependent kinase. In this study we report that K252a inhibits both PIP_2-dependent and PIP_2-independent routes of respiratory burst activation. These results are consistent with active participation of PKC in both cases.

MATERIALS AND METHODS

Preparation of neutrophils

Human blood was collected by venipuncture and neutrophils prepared by Ficoll–Isopaque separation as described [6]. Cell purity was greater than 97% and viability greater than 99% (Trypan Blue exclusion).

Quin 2 assay

Cytosolic Ca^{2+} levels were measured by use of the

* To whom correspondence should be addressed.

† Abbreviations: O_2^-, superoxide; PIP_2, phosphatidylinositol bisphosphate; IP_3, inositol triphosphate; $[Ca^{2+}]_i$, intracellular calcium concentration; DAG, diacylglycerol; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; fMLP, formyl-methionyl-leucyl-phenyl-alanine; HBSS, Hank's balanced salt solution: Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; PBS, phosphate buffered saline; PA, phosphatidic acid; PC, phosphatidylcholine; PLC, phospholipase C; PLD, phospholipase D; PLA_2, phospholipase A_2.

fluorescent indicator Quin 2 [10, 15]. To deplete the cells of Ca^{2+}, neutrophils were suspended in Ca^{2+}-free Hank's balanced salt solution (HBSS), pH 7.4, containing 1 mM ethyleneglycolbis(aminoethyl-ether)tetra-acetate (EGTA) (the Sigma Chemical Co., St Louis, MO) and 40 μM Quin 2/AM (Calbiochem, La Jolla, CA), at a concentration of 5×10^7 cells/mL, and incubated at 37° for 60 min. Normal Ca^{2+} levels were measured after incubating in HBSS containing 0.5 mM Ca^{2+}, 1 mM Mg^{2+} and 10 μM Quin 2/AM, at 2.5×10^7 cells/mL for 15 min, diluted to 5×10^6 cells/mL for the remaining 45 min. To check for autofluorescence, 5×10^6 cells/mL were incubated in HBSS with 0.5 mM Ca^{2+} and 1 mM Mg^{2+} alone.

After 60 min at 37°, cells were washed twice in their respective media and suspended in a standard Tyrode solution consisting of: 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mg/mL glucose and buffered with 20 mM Hepes at pH 7.4. To this was added either 1 mM EDTA or 3 mM Ca^{2+} and 1 mM Mg^{2+}. To obtain minimum and maximum fluorescence readings, 0.1% Triton X-100 was added to the above solutions.

Fluorescence was measured in a Perkin-Elmer 3000 spectrofluorimeter at excitation and emission wavelengths of 339 and 492 nm respectively, using a stirring cuvette for all readings. The values obtained from those cells not loaded with Quin 2 were subtracted from all other readings, and $[Ca^{2+}]_i$ calculated using the equation: $K_d(F - F_{min})/(F_{max} - F)$, where K_d, the dissociation constant of Ca^{2+} binding to Quin 2, is 115 nM.

Superoxide assay

ConA-zymosan activation. ConA-zymosan was prepared by boiling zymosan (the Sigma Chemical Co., St Louis, MO) in saline for 30 min, washing twice, then incubating for 30 min at 37°, at a final concentration of 10 mg/mL, with 0.5 mg/mL con A (the Sigma Chemical Co.), in HBSS containing Ca^{2+}. Finally, the ConA-zymosan was washed and resuspended in Ca^{2+}-free HBSS to the appropriate concentrations.

Neutrophils loaded with Quin 2, in the presence or absence of Ca^{2+}, were washed twice then resuspended in Tyrode solution containing 1 mg/mL bovine serum albumin (Miles Laboratories). One mM EGTA was added to Ca^{2+}-depleted cells. 2×10^6 cells were then aliquoted into assay tubes containing 1 mg ferricytochrome *c* (horse heart type III, the Sigma Chemical Co.), K252a or Tyrodes, ConA-zymosan or Tyrodes and either 75 units superoxide dismutase (bovine blood, the Sigma Chemical Co.) or Tyrodes solution. All reagents were prepared in the respective Tyrode solutions, with or without EGTA. Ca^{2+} was added back to relevant tubes at a final concentration of 3 mM.

The reaction was terminated after 30 min at 37° by the addition of 1 mM *N*-ethylmaleimide (the Sigma Chemical Co.). Following centrifugation at 1400 *g* for 10 min, at 4°, absorbance of the supernatant was read at 550 nm in a Beckman DU-50 spectrophotometer. The amount of O_2^- produced was calculated by dividing the difference in absorbance of the samples, with and without superoxide dismutase, by the extinction coefficient for the change between ferricytochrome *c* and ferrocytochrome *c* ($E_{550} = 15.5\ mM^{-1}\ cm^{-1}$) and the resulting value converted to nmol O_2^- per 5×10^6 cells. Results are expressed as a percentage of the maximum control response (i.e. in the absence of inhibitor).

PMA-fMLP, PMA-fluoride activation. For phorbol myristate acetate (PMA) and formyl-methionyl-leucyl-phenyl-alanine (fMLP), Tyrodes solution containing 1 mg/mL bovine serum albumin was employed. A modified Dulbecco's phosphate buffered saline (PBS) was used for all fluoride experiments, containing 136.9 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 0.31 mM $CaCl_2$, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, in which the concentration of NaCl was reduced so that the final salt concentration was physiological after the addition of NaF.

Neutrophils were prepare as described and equilibrated at 37° for 20 min. Cytochalasin B (5 μg/mL) (the Sigma Chemical Co.) was added to those cells which were to be stimulated with fMLP and 2×10^6 cells then immediately dispensed into tubes containing ferricytochrome *c* and superoxide dismutase, as described, and K252a or Tyrodes. Incubation was continued for a further 5 min before adding stimulus to start the reaction. Where sequential PMA-fMLP or PMA-fluoride activation occurred, cells were pretreated with PMA (0.5 ng/mL) (the Sigma Chemical Co.) for 3 min before challenging with K252a for a further 3 min, prior to adding either fMLP (the Sigma Chemical Co.) or fluoride (BDH, Poole, U.K.). The reaction then proceeded as detailed for ConA-zymosan activation.

K252a was a generous gift from Dr Yamada of the Tokyo Research Laboratories, Kyowa Hakko Kogyo Co. Ltd, Machida-shi, Tokyo 194, Japan.

RESULTS

PMA-primed neutrophils

In three experiments, neutrophils primed with non-stimulatory doses of PMA (0.5 ng/mL) released greater amounts of O_2^- on subsequent activation with fMLP than with fMLP alone (Fig. 2A), the concentration–response curve for fMLP being displaced to the left and the maximum response increased. This also occurs with the fluoride-mediated concentration–response curve (Fig. 2B). K252a, at 1 and 0.2 μM, caused complete inhibition of the respiratory burst to fMLP and fluoride respectively, whether given alone or after PMA-priming (Fig. 2A and B).

Ca^{2+}-depleted neutrophils

In three experiments the mean resting $[Ca^{2+}]_i$ in normal control neutrophils, recorded by Quin 2 fluorescence, was 125.09 ± 3.97 nM, whereas in cells subjected to the Ca^{2+} depletion regime, the mean resting $[Ca^{2+}]_i$ was 20.86 ± 4.04 nM.

K252a (1 μM) inhibited the response to ConA-zymosan in both normal and Ca^{2+}-depleted neutrophils as depicted in Fig. 3A, shifting the concentration–response curve to the right and markedly depressing the maximum response. Superoxide production was reduced in Ca^{2+}-depleted neutrophils and this was further reduced by the addition of K252a.

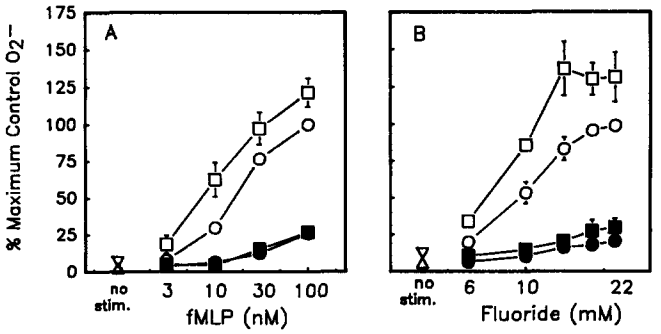

Fig. 2. The effect of K252a on the O_2^- dose–response curves of fMLP and fluoride: (A) fMLP control (○) and in the presence of K252a, 1 μM (●). fMLP in cells primed with a non-stimulatory dose of PMA (0.5 ng/mL), alone (□) and in the presence of K252a, 1 μM (■) (N = 3). (B) Fluoride, control (○) and in the presence of K252a, 0.2 μM (●). Fluoride in cells primed with PMA (0.5 ng/mL), alone (□) and with K252a, 0.2 μM (■) (N = 3). In both (A) and (B) (△) represents cells alone and (▽) in the presence of 0.5 ng/mL PMA. The mean maximum O_2^- release, expressed as nmol $O_2^-/5 \times 10^6$ neutrophils, was 141.10 ± 15.75 for fMLP and 97.02 ± 11.76 for fluoride.

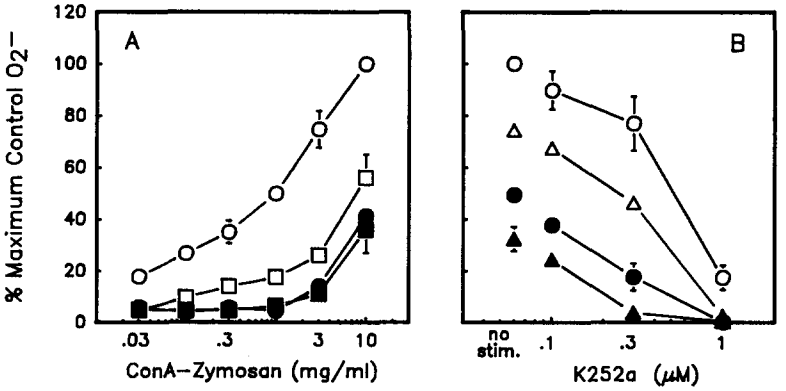

Fig. 3. (A) The effect of K252a and Ca^{2+}-depletion on the O_2^- dose–response curve of ConA-zymosan: For normal cells, ConA-zymosan in the presence (●) and absence (○) of K252a, 1 μM. For Ca^{2+}-depleted cells, ConA-zymosan in the presence (■) and absence (□) of K252a, 1 μM (N = 3). The mean maximum O_2^- release, expresed as nmol $O_2^-/5 \times 10^6$ neutrophils, was 68.74 ± 8.49 for normal cells. (B) The effect of a range of K252a concentrations on O_2^- generated by ConA-zymosan in the presence and absence of Ca^{2+}: For normal cells, ConA-zymosan 10 mg/mL (○) and 3 mg/mL (●). For Ca^{2+}-depleted cells, ConA-zymosan 10 mg/mL (△) and 3 mg/mL (▲) (N = 3). The mean 100% control O_2^- release, expressed as nmol $O_2^-/5 \times 10^6$ neutrophils, was 114.52 ± 7.54 for normal cells with 10 mg/mL ConA-zymosan. In normal cells the mean resting Ca^{2+} concentration was 125.09 ± 3.97 nM; in Ca^{2+}-depleted cells the mean resting Ca^{2+} concentration was 20.86 ± 4.04 nM.

Using concentrations of ConA-zymosan which gave measureable release of O_2^- in both the presence and absence of Ca^{2+}, namely 3 and 10 mg/mL, a dose–inhibition curve with K252a was constructed (Fig. 3B). This shows the inhibitory effect of K252a to be dose-related and to be virtually maximal at 1 μM in each case.

DISCUSSION

Two distinct, novel activation mechanisms for the neutrophil oxidative burst have been described, both reported as being independent of PIP_2 turnover, and one reported as not requiring PKC.

Firstly, with fMLP as the simulus, prior exposure to subthreshold PMA was reported to initiate a transduction pathway in which there was decreased or absent PIP_2 turnover and little or no increase in $[Ca^{2+}]_i$—in contrast to the non-primed system—although the subsequent response to the agonists was considerably greater than in the control cells not primed with PMA [7]. In addition, it has been shown that the respiratory burst stimulated by either fluoride or fMLP, is prevented by prior Ca^{2+}-depletion of the neutrophils, but can be restored by priming the Ca^{2+}-depleted cells with subthreshold doses of PMA [8, 9], although the restored burst is not accompanied by PIP_2 turnover, or PKC translocation.

In these experiments, some degree of PKC activation was clearly necessary because of the requirement for PMA. The question therefore arises as to what transduction mechanism was involved in the subsequent fMLP- or fluoride-induced superoxide release. It has been shown that priming with PMA before fMLP stimulation results in more DAG production than occurs with fMLP alone, that this new DAG comes from a non-PIP_2 source and that the sequence of events is blocked by sphinganine [16]. Furthermore, there have been a number of studies proposing that phosphatidylcholine (PC) could act as a non-PIP_2 source of DAG [17–19]. In the present study the IP_3/Ca^{2+}-independent activation sequence, involving non-stimulatory PMA followed by fMLP or fluoride, is fully inhibited by K252a, at the same concentration that inhibits the PIP_2/Ca^{2+}-dependent pathway. (It should be noted that because of the timing of the addition of K252a it is the second activation of PKC which is inhibited.) It is somewhat surprising that K252a caused almost complete inhibition of the fluoride-induced superoxide response in both primed and unprimed cells at a concentration of 0.2 μM which is less than the K_i values of 0.27 and 0.58 μM recorded against the isolated enzyme. K252a is a competitive inhibitor at the ATP substrate binding site and intracellular ATP concentrations are much higher (of the order 1 mM) compared to those in *in vitro* enzyme assays. One explanation for this phenomenon is that fluoride can cause a decrease in the intracellular concentrations of ATP [20] such that the inhibitor concentration required to block PKC activity would be much reduced. Alternatively, fluoride may recruit a particular PKC isoenzyme that is more sensitive to inhibition by K252a (discussed in Ref. 13). These results, like those quoted above [16], are consistent with PKC being involved in this PIP_2-independent signal transduction mechanism.

However, since the priming dose of PMA does not, in itself, cause O_2^- generation, there is still the question as to how subsequent receptor stimulation (as with fMLP) or G-protein stimulation (as with fluoride) triggers a DAG/PKC pathway. There is evidence that a pathway exists involving the fMLP receptor and a phospholipase D which acts on PC giving rise to DAG [21]. A PC source of DAG produced by phospholipase D (PLD) action could also be involved [22–24] and there is evidence that this pathway is implicated in the transduction of the neutrophil oxidative burst [25, 26]. However, the possibility that arachidonate may play a part cannot be ruled out. Arachidonate is known to be a PKC activator [27] and phospholipase A_2 (PLA_2), which gives rise to arachidonate, can be activated by receptor stimulation [28, 29]. We have recently reported data which, on one interpretation, could mean that the respiratory burst induced by IgG and opsonised zymosan is mediated in part by arachidonate [30]. Is this the PIP_2-independent pathway in PMA-primed cells?

A second novel activation mechanism recently described [10] involved a ConA-zymosan-induced respiratory burst in Ca^{2+}-depleted neutrophils, without any accompanying turnover of phosphoinositides, or increase in $[Ca^{2+}]_i$ or arachidonate release; there was no pretreatment with PMA and the burst (like the burst in control cells) was not inhibited by H-7. The conclusion was that the activation was not only PIP_2-independent, but that PKC was not involved. The data reported showed quite clearly that the response was, in fact, PIP_2-independent. However, the results do not necessarily indicate that PKC was not involved. In the first place the results with H-7 are not conclusive. H-7 has been described as a non-specific PKC inhibitor [31]. Not only have results reported with H-7 on the normal neutrophil oxidative burst been contradictory [32–34], but quite different results have been obtained with other PKC inhibitors such as sphinganine [16] and K252a [13, 35]. More importantly, H-7 in a recent study failed to inhibit cellular events induced by phorbol esters, agents which specifically stimulate PKC [36]. In fact, it might be said that H-7 is unsuitable as a tool to probe for PKC involvement in signalling processes.

In the present study K252a, which is reported to be a more selective PKC inhibitor [13, 14], produced a dose-related reduction of the ConA-zymosan-mediated burst in both normal and Ca^{2+} depleted neutrophils, eliminating it entirely at 1 μM. To the extent that K252a is specific for PKC, this implies that PKC activation is involved in the burst. The question then arises as to what the endogenous PKC activator could be in this particular system. DAG produced by PLD action is clearly not involved, since it was shown that there was no increase in PA in this activation sequence [10]. It was also reported in this latter study that the ConA-zymosan-induced burst was insensitive to a pertussis toxin. It is possible that DAG could be generated from phosphatidylinositol by a non-G-protein-sensitive PLC, and that this DAG is metabolised not by the DAG kinase pathway which leads to PA and the recycling of the phosphoinositides, but by DAG lipase.

The possibility that arachidonate is the PKC activator, suggested above, is not really ruled out by the data reported [10]. The role of endogenous arachidonate in stimulus-activation coupling mechanisms is difficult to investigate rigorously. Techniques involving the use of radioactive arachidonate have several serious drawbacks [discussed in 37]. One is that several distinct pools of arachidonate exist, and the arachidonate which may function as an intracellular messenger could be in a pool which is not released to the exterior. Another is that there may be rapid reacylation of the key phospholipids which are transiently deacylated during activation of the cell. Thus, the lack of exterior release of radiolabelled arachidonate from Ca^{2+}-depleted ConA-zymosan-stimulated neutrophils does not necessarily indicate that arachidonate is not involved in transduction events. PLA_2, a Ca^{2+}-dependent enzyme, could still function in cells which are notionally "Ca^{2+}-depleted" since some Ca^{2+} is clearly still present, and it is possible that Ca^{2+}, like arachidonate, exists in distinct pools which are not necessarily all depleted by the Ca^{2+}-depleting techniques employed. Arachidonate could also be generated by the DAG lipase pathway.

To the extent that K252a is a specific PKC

inhibitor, our results with K252a in the present study indicate that, which ever pathway is involved in PIP_2/Ca^{2+}-independent transduction, PKC appears to be necessary for O_2^- generation with fMLP, fluoride or ConA-zymosan, since in the presence of this inhibitor the burst is totally eradicated. It could be argued that the effects of K252a obtained in this study could be due to inhibition of two different protein kinases, namely PKC in the PMA-primed system and another protein kinase in the non-primed system; however, this would appear to be unlikely since the same concentration of K252a almost totally inhibits both systems. The fact that not only K252a but also staurosporine [13] and three other, recently described PKC inhibitors [38] are all effective inhibitors in the non-primed system would tend to indicate that PKC could well be involved in this as well as in the PMA-primed system.

In conclusion, our results are consistent with the proposal that, both in the physiological situation where there is PIP_2 turnover with increased $[Ca^{2+}]$ and [DAG], and in the PIP_2-independent pathway (whatever the activation mechanism), PKC has a role in the generation of a respiratory burst. This is in agreement with a previous study where both K252a and staurosporine have been shown to cause an inhibition of the respiratory burst induced by six different stimuli [13].

Acknowledgements—This work was supported by grants from the Emily le Rossignol Fund, UCL, and the Arthritis and Rheumatism Council. We would like to thank Mrs Joyce Mancini for her excellent technical assistance and Dr Yamada of the Tokyo Research Laboratories, Kyowa Hakko Kogyo Co. Ltd., Machida-shi, Tokyo 194, Japan for the generous gift of K252a. We also wish to thank Robert Muid for his help and advice during the preparation of this manuscript.

REFERENCES

1. Tate RM and Repine JE, Phagocytes, oxygen radicals and lung injury. In: *Free Radicals in Biology* (Ed. Pryer WA), Vol. VI, pp. 199–209. Academic Press, New York, 1984.
2. Nishizuka Y, Role of protein kinase C in cell surface signal transduction and cyclic nucleotide-dependent protein kinases. *Nature* **308**: 693–698, 1984a.
3. Berridge MJ and Irvine RF, Inositol triphosphate, a novel second messenger in cellular signal transduction. *Nature* **312**: 315–321, 1984.
4. Robinson JM, Badwey JA, Karnovsky ML and Karnovsky MJ, Superoxide release by neutrophils: Synergistic effects of a phorbol ester and a calcium ionophore. *Biochem Biophys Res Commun* **122**: 734–739, 1984.
5. Dale MM and Penfield A, Synergism between phorbol ester and A23187 in superoxide production by neutrophils. *FEBS Lett* **175**: 170–172, 1984.
6. Penfield A and Dale MM, Synergism between A23187 and 1-oleoyl-2-acetyl-glycerol in superoxide production by human neutrophils. *Biochem Biophys Res Commun* **125**: 332–336, 1984.
7. Della Bianca V, Grzeskowiak M, Cassatella MA, Zeni L and Rossi F, Phorbol 12, myristate 13, acetate potentiates the respiratory burst while inhibits phosphoinositide hydrolysis and calcium mobilisation by formyl-methionyl-leucyl-phenylalanine in human neutrophils. *Biochem Biophys Res Commun* **135**: 556–565, 1986.
8. Grzeskowiak M, Della Bianca V, Cassatella MA and Rossi F, Complete dissociation between the activation of phosphoinositide turnover and of NADPH oxidase by formyl-methionyl-leucylphenylalanine in human neutrophils depleted by Ca^{2+} and primed by subthreshold doses of phorbol 12, myristate 13, acetate. *Biochem Biophys Res Commun* **135**: 785–794, 1986.
9. Della Bianca V, Grzeskowiak M, Dusi S and Rossi F, Fluoride can activate the respiratory burst independently of Ca^{2+}, stimulation of phosphoinositide turnover and protein kinase C translocation in primed human neutrophils. *Biochem Biophys Res Commun* **150**: 955–964, 1988.
10. Rossi F, Della Bianca V, Grzeskowiak M and Bazzoni F, Studies on molecular regulation of phagocytosis in neutrophils: ConA-mediated ingestion and associated respiratory burst independent of phosphoinositide turnover, rise in $[Ca^{2+}]_i$, and arachidonic acid release. *J Immunol* **142**: 1652–1660, 1989.
11. Kase H, Iwahashi K, Nakanishi S, Matsuda Y, Yamada K, Takahashi M, Murakata C, Sato A and Kaneko M, K252a compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochem Biophys Res Commun* **142**: 436–440, 1987.
12. Davis PD, Hill CH, Keech E, Lawton G, Nixon JS, Sedgwick AD, Wadsworth J, Westmacott D and Wilkinson S, Potent selective inhibitors of protein kinase C. *FEBS Lett* **259**: 61–63, 1989.
13. Twomey B, Muid RE and Dale MM, The effect of protein kinase C inhibitors, K252a and staurosporine, on the human neutrophil respiratory burst activated by both receptor stimulation and post-receptor mechanisms. *Br J Pharmacol* **100**: 819–825, 1990.
14. Smith RJ, Justen JM and Sam LM, Effects of a protein kinase *C* inhibitor, K252a, on human polymorphonuclear neutrophil responsiveness. *Biochem Biophys Res Commun* **152**: 1497–1503, 1988.
15. Tsien RY, Pozzan T and Rink TJ, T-cell mitogens cause early changes in cytoplasmic free Ca^{2+} and membrane potential in lymphocytes. *J Cell Biol* **94**: 325–334, 1982.
16. Tyagi SR, Tamura M, Burnham DN and Lambeth JD, Phorbol myristate acetate (PMA) augments chemoattractant-induced diglyceride generation in human neutrophils but inhibits phosphoinositide hydrolysis. *J Biol Chem* **263**: 13191–13198, 1988.
17. Daniel LW, Waite M and Wykle RL, A novel mechanism of diglyceride formation. *J Biol Chem* **261**: 9128–9132, 1986.
18. Besterman JM, Duronio V and Cuatrecasas P, Rapid formation of diglyceride from phosphatidylcholine: A pathway for generation of a second messenger. *Proc Natl Acad Sci USA* **83**: 6785–6789, 1986.
19. Reibman J, Korchak HM, Vosshall LB, Haines KA, Rich AM and Weissmann G, Changes in diglyceride labelling, cell shape, and protein phosphorylation distinguish "triggering" from "activation" of human neutrophils. *J Biol Chem* **263**: 6322–6328, 1988.
20. Svec F, Relationship between intact cell ATP levels and glucocorticoid receptor-binding capacity in the AtT-20 cell. *Biochim Biophys Acta* **847**: 147–154, 1985.
21. Truett A, Snyderman R and Murray JJ, Stimulation of phosphorylcholine turnover and diacylglycerol production in human polymorphonuclear leukocytes: Novel assay for phosphorylcholine. *Biochem J* **260**: 909–913, 1989.
22. Cabot MC, Welsh CJ, Cao H and Chabbot H, The phosphatidylcholine pathway of diacylglycerol formation stimulated by phorbol diesters occurs via phospholipase D activation. *FEBS Lett* **263**: 153–157, 1988.
23. Gelas P, Ribbes G, Record M, Terce F and Chap H,

Differential activation by fmet-leu-phe and phorbol ester of a plasma membrane phosphatidylcholine-specific phospholipase D in human neutrophils. *FEBS Lett* **251**: 213–218, 1989.
24. Agwu DE, McPhail LC, Chabot MC, Daniel LW, Wykle RL and McCall CE, Choline-linked phosphoglycerides: A source of phosphatidic acid and diglycerides in stimulated neutrophils. *J Biol Chem* **264**: 1405–1413, 1989.
25. Bonser RW, Thompson NT, Randall RW and Garland LG, Phospholipase D activation is functionally linked to superoxide generation in the human neutrophil. *Biochem J* **264**: 617–620, 1989.
26. Reinhold SL, Prescott SM, Zimmerman GA and McIntyre TM, Activation of human neutrophil phospholipase D by three separable mechanisms. *FASEB J* **4**: 208–214, 1990.
27. Dreher ML and Hanley MR, Multiple modes of protein kinase C regulation and their significance in signalling. *Trends Pharmacol Sci* **9**: 114–115, 1988.
28. Dahinden CA, Zingg J, Maly FE and De Weck AL, Leukotriene production in human neutrophils primed by recombinant human granulocyte/macrophage colony-stimulating factor and stimulated with the complement component C5A and fMLP as second signals. *J Exp Med* **167**: 1281–1295, 1988.
29. Axelrod J, Burch RM and Jelsema CL, Receptor-mediated activation of phospholipose A_2 via GTP-binding proteins: Arachidonic acid and its metabolites as second messengers. *Trends Neurol Sci* **11**: 117–123, 1988.
30. Muid RE, Twomey B and Dale MM. The effect of the inhibition of both diacylglycerol metabolism and phospholipase A_2 activity on superoxide generation by human neutrophils. *FEBS Lett* **234**: 235–240, 1988.
31. Garland LG, Bonser RW and Thompson NT, Protein kinase C inhibitors are not selective. *Trends Pharmacol Sci* **8**: 334, 1987.
32. Wright CD and Hoffman MD. The protein kinase C inhibitors H-7 and H-9 fail to inhibit human neutrophil activation. *Biochem Biophys Res Commun* **135**: 749–755, 1986.
33. Fujita I, Takeshige K and Minakami S, Inhibition of neutrophil superoxide formation by 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7). *Biochem Pharmacol* **35**: 4555–4562, 1986.
34. Seifert R and Schachtele C, Studies with protein kinase C inhibitors presently available cannot elucidate the role of protein kinase C in the activation of NADPH oxidase. *Biochem Biophys Res Commun* **152**: 585–592, 1988.
35. Smith RJ, Justen JM and Sam LM, Effects of a protein kinase C inhibitor, K252a, on human polymorphonuclear neutrophil responsiveness. *Biochem Biophys Res Commun* **152**: 1497–1503, 1988.
36. Nixon JS, Wilkinson SE, Davis PD, Sedgwick AD, Wadsworth J and Westmacott D, Inhibition of cellular processes by H-7. *Agents Actions*, in press.
37. Irvine RF. How is the level of free arachidonic acid controlled in mammalian cells? *Biochem J* **204**: 3–16, 1982.
38. Twomey B, Muid RE, Nixon JS, Sedgwick AD, Wilkinson SE and Dale MM, The effect of new potent selective inhibitors of protein kinase C on the neutrophil respiratory burst. *Biochem Biophys Res Commun* **171**: 1087–1092, 1990.