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A study on the role of protein kinase C and intracellular calcium in the activation of superoxide generation

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Accumulating evidence indicates that protein kinase C plays an essential role in the activation of NADPH oxidase. In the present study, the correlation between superoxide generation, intracellular calcium, activation of purified protein kinase C and stabilized membrane-bound protein kinase C was studied. Phorbol 12-myristate 13-acetate (PMA) and 1-deacyl-2-acetyl-*rac*-glycerol (OAG) were found to induce equal activation of purified protein kinase C and translocation of protein kinase C to the membrane fraction, but differed significantly in their ability to induce superoxide generation. Intracellular calcium was varied using calcium ionophores and increasing the intracellular calcium concentration to more than 1 μM was found to induce increased superoxide generation in maximally OAG-stimulated cells; this contrasted to maximally PMA-stimulated leukocytes. Ionomycin and A23187 were both found to induce a translocation of protein kinase C to the membrane fraction. This translocation was highly dependent upon extracellular calcium. In contrast, PMA- and OAG-induced translocation of protein kinase C was not dependent upon extracellular calcium. In conclusion, our results indicate that although PMA, OAG and calcium ionophores seem to activate protein kinase C in human polymorphonuclear leukocytes these activators differ in their ability to induce superoxide generation.

Introduction

Superoxide generation is an essential component of the neutrophil bactericidal machinery. NADPH oxidase is supposed to constitute the main enzyme responsible for superoxide generation, but the mechanism whereby NADPH oxidase is activated remains unknown. Accumulating evidence indicates that the calcium- and phospholipid-dependent protein kinase, protein kinase C, plays an essential role in the activation of NADPH oxidase. This hypothesis is mainly based on the following observations: (1) stimulation of superoxide generation by protein kinase C activators [1,2]; (2) phosphorylation of NADPH oxidase by purified protein kinase C [3]; and (3) activation of NADPH oxidase by purified protein kinase C [4].

The association of protein kinase C with the cell membrane observed upon stimulation with some activators is proposed to be essential to protein kinase C activation [5]. It has been con-

Abbreviations: PMA, phorbol 12-myristate 13-acetate; OAG, 1-oleoyl-2-acetyl-*rac*-glycerol; FMLP, *N*-formylmethionylleucylphenylalanine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylene diaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid.

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cluded that there are at least two types of protein kinase C association with the cell membrane. One type, reversible calcium-induced membrane binding, can be dissociated by chelators. A second type, PMA-induced (stabilized) membrane binding, is stable to chelators and high salt concentrations, but can be dissociated with detergents [6]. The significance of these two types of membrane association remains a matter of debate.

Receptor-induced activation of protein kinase C is proposed to involve phosphatidylinositol hydrolysis resulting in an increased diacylglycerol formation and, thereby, protein kinase C activation and inositol trisphosphate formation inducing an elevation of intracellular calcium [7]. In vitro, this system is mimicked by phorbol ester activation of protein kinase C and Ca^{2+} elevation induced by ionophores [7]. This artificial model was used in the present study to compare superoxide generation, intracellular calcium, activation of purified protein kinase C and stabilized membrane-bound protein kinase C.

Materials and Methods

The calcium ionophore ionomycin was obtained from Calbiochem. Other chemicals were obtained as described [8–10].

Buffer solutions. Buffer A comprised 140 mM NaCl/2.7 mM KCl/8 mM Na_2HPO_4/1.4 mM KH_2PO_4/1 mM $MgCl_2$/0.6 mM $CaCl_2$ (pH 7.5). Buffer B comprised 20 mM Tris-HCl (pH 7.5)/0.5 mM EGTA/0.5 mM EDTA/1 mg/ml soybean trypsin inhibitor/1 mM benzamidine/1 mM dithiothreitol. Buffer C was 10 mM Hepes (pH 7.4)/145 mM NaCl/5 mM KCl/1 mM Na_2HPO_4/0.5 mM $MgSO_4$/5 mM glucose/1 mM $CaCl_2$.

Assay for superoxide production. Superoxide production was assayed as superoxide dismutase-inhibitable reduction of cytochrome *c*. Human polymorphonuclear leukocytes isolated as in Refs. 11 and 12 were transferred to 1 ml buffer A or buffer C containing 1 mg/ml cytochrome *c*, PMA or OAG as indicated. The incubation was continued for 10 min in the presence or absence of 30 μg/ml superoxide dismutase. The incubation was stopped by addition of 10 μl 3 mg/ml superoxide dismutase to the sample without superoxide dismutase and the absorbance measured at 550 nm.

Assay of protein kinase C. Protein kinase C was assayed for 10 min in the absence or presence of phosphatidylserine and diolein [9].

Ca^{2+} dependence of purified protein kinase C. Protein kinase C was purified as described in Ref. 9. A mixture of the following components was made: 180 μl 200 mM Tris-HCl (pH 7.5) containing 80 mg/ml histone III-S, 360 μl 14 mM EGTA buffered with variable amounts of $CaCl_2$ and 720 μl purified protein kinase C (0.25 U/ml). To this mixture was added 360 μl of 20 mM Tris-HCl (pH 7.5) containing either phosphatidylserine (240 μg/ml), OAG (0.6 mg/ml) or PMA (6 μg/ml) as described in Fig. 4. Finally, the reaction was started by adding 180 μl of a solution containing 600 μM ATP and 60 mM magnesium acetate and was monitored by withdrawing (at 15 s) 100 μl, which was added to 20 μl tracer amounts of [γ-^{32}P]ATP ($(1–2) \cdot 10^7$ cpm/ml) in 20 mM Tris-HCl (pH 7.5). This radioactive phosphorylation reaction was terminated after incubation for 10 min at 30 °C. 75 μl of the reaction mixture were spotted on phosphocellulose paper and the paper was washed as described [8].

To the remaining 1700 μl non-radioactive reaction mixture, 340 μl 20 mM Tris-HCl (pH 7.5) were added and the free Ca^{2+} concentrations were measured using the calcium indicator quin2 as described below.

Assay for translocation of protein kinase C activity. Human polymorphonuclear leukocytes were isolated as described in Ref. 12 and preincubated for 10 min in 2 ml buffer A at 37 °C and subsequently activated by PMA, OAG, FMLP, A23187 and ionomycin as indicated in the figure legends. The activation period was stopped after 10 min by addition of 5 ml ice-cold buffer A and the cells were sedimented by centrifugation at $200 \times g$ for 10 min. Finally, the cells were resuspended in 2 ml buffer B and ultrasonically disrupted. Preparation of the particulate fraction, Triton X-100 solubilization and DEAE-cellulose chromatography of solubilized protein kinase C were carried out as previously described [10]. The amount of protein kinase C translocated was expressed as a fraction of cytoplasmic protein kinase C activity in unstimulated cells.

Determination of intracellular calcium. Human polymorphonuclear leukocytes were resuspended in buffer C at a concentration of $5 \cdot 10^7$ cells/ml. The cells were preincubated in a 37°C waterbath for 5 min and 20 μM quin2/AM was added. After 20 min, the cell suspension was diluted 10-fold with 37°C warm buffer C and the incubation was continued for another 40 min. Finally, the cells were washed, resuspended in buffer C at a concentration of $2 \cdot 10^7$ cells/ml and kept at room temperature until use. Immediately before use, $2 \cdot 10^7$ cells were centrifuged and resuspended in 2 ml 37°C buffer C in a thermostated fluorometer cuvette with constant stirring. Fluorescence was measured with a Perkin-Elmer luminiscencemeter LS-5 (excitation, 339 nm; emission, 492 nm). F_{max} was obtained by addition of 50 μM digitonin and F_{min} was obtained by addition of 1 mM $MnCl_2$. Free cytoplasmic calcium was calculated from:

$$[Ca^{2+}]_i = 115 \text{ nm} \cdot \frac{F - F_{min}}{F_{max} - F}$$

Determination of free calcium in buffer solutions. 2 ml buffer solution was placed in a thermostated fluorometer cuvette and 1 μM quin2 (free acid) was added. Fluorescence was measured as described above. F_{max} was obtained by addition of 1 μM $CaCl_2$ and F_{min} was obtained by addition of 1 mM $MnCl_2$. Free calcium was calculated from:

$$[Ca^{2+}] = K_d \cdot \frac{F - F_{min}}{F_{max} - F}$$

where K_d is the dissociation constant of the quin2-Ca complex corrected for Mg^{2+} concentration according to Ref. 13.

Results

Superoxide generation and protein kinase C translocation in PMA- and OAG-stimulated neutrophils

Both PMA and OAG were found to induce superoxide generation in human polymorphonuclear leukocytes and at a cell concentration of $1 \cdot 10^6$ leukocytes/ml, 1 μg/ml PMA and 0.1 mg/ml OAG induced a maximal effect upon superoxide generation. However, the maximal effect of PMA was at least 10-times greater than that of OAG (Table I).

TABLE I

SUPEROXIDE GENERATION AND TIGHT MEMBRANE-ASSOCIATED PROTEIN KINASE C ACTIVITY IN PMA- AND OAG-STIMULATED NEUTROPHILS

Neutrophils were isolated as in Ref. 12 and preincubated for 10 min in buffer A. To measure superoxide generation, the neutrophils were diluted to $1 \cdot 10^6$ neutrophils/ml in buffer A containing 1 mg/ml cytochrome *c* and PMA, OAG or buffer A, and the incubation was continued for 10 min the presence or absence of superoxide dismutase. Reduction of cytochrome *c* was measured at 550 nm. To measure membrane-associated protein kinase C, $13 \cdot 10^6$ neutrophils were diluted to $1 \cdot 10^6$ leukocytes/ml and incubated for 10 min at 37°C in the presence of PMA or OAG, and membrane-associated protein kinase C was measured as described. Membrane-associated protein kinase C and superoxide generation were measured in parallel experiments. One representative experiment of four is shown.

Neutrophil stimulators	Particulate fraction-associated protein kinase activity (mU/10^6 cells ± S.E., $n = 3$)	Superoxide production (nmol/10 min per 10^6 cells ± S.E., $n = 3$)
None	19 ± 2	3.6 ± 0.9
PMA (1 μg/ml)	147 ± 30	51 ± 0.8
OAG (0.1 mg/ml)	198 ± 10	6.6 ± 0.6

In unstimulated leukocytes, only a few percent of the total protein kinase C activity was found to be associated to the cell membrane. Stimulation of the neutrophils by PMA or OAG induced a severalfold increase in particulate fraction-associated protein kinase C activity (Table I). When $1 \cdot 10^6$ leukocytes/ml were stimulated by 0.1 mg/ml OAG, the particulate fraction associated-protein kinase C activity increased to approx. 70% of the total protein kinase C activity. Using 1 μg/ml PMA, a comparable increase (50%) in membrane-associated protein kinase C activity was found (Table I). As the differences in superoxide generation could have been explicable by a different rate of translocation of protein kinase C, we compared the relative amount of translocated protein kinase C in neutrophils stimulated by PMA or OAG for 2–20 min and found no differences in the rate of translocation (data not shown).

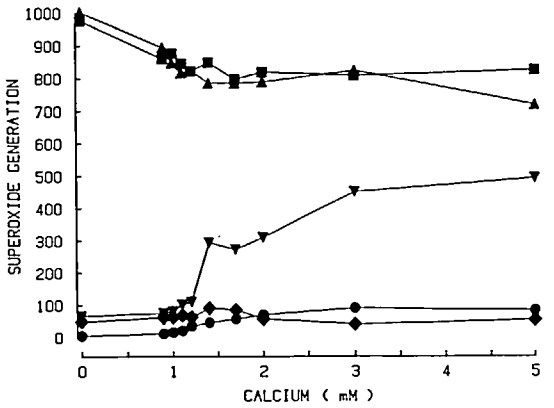

Fig. 1. Calcium activation of superoxide generation in OAG- and PMA-activated neutrophils prestimulated by A23187. $1 \cdot 10^7$ neutrophils/ml were isolated as in Ref. 11 and preincubated for 10 min at 37°C in buffer C containing 1 mM EGTA and calcium as indicated, followed by a further preincubation for 5 min with or without 2.5 μg/ml A23187. To measure superoxide generation, the neutrophils were diluted to $0.5 \cdot 10^6$/ml and the superoxide dismutase-inhibitable reduction of cytochrome *c* was determined, as in Table I, and expressed as the difference in absorbance at 550 nm ($\times 10^3$). The symbols indicate: ●, preincubated in the presence of 2.5 μg/ml A23187 and incubated without further additions; ▼, preincubated in the presence of 2.5 μg/ml A23187 and stimualted by OAG 0.1 mg/ml; ▲, preincubated in the presence of 2.5 μg/ml A23187 and stimulated by 1 μg/ml PMA; ◆, preincubated in the absence of A23187 and stimulated by OAG 0.1 mg/ml; ■, preincubated in the absence of A23187 and incubated in the presence of PMA 1 μg/ml.

Calcium activation of superoxide generation in OAG- and PMA-stimulated neutrophils

Superoxide generation was studied in PMA- and OAG-activated leukocytes varying intracellular calcium with ionomycin and A23187. As the calcium ionophore A23187 was fluorescent by itself, intracellular calcium could only be monitored by quin2 in ionomycin-stimulated leukocytes. Superoxide generation was measured using constant concentrations of EGTA, A23187, OAG or PMA and increasing extracellular calcium. In OAG-activated cells, superoxide generation was low and unaffected by increasing extracellular calcium concentrations (Fig. 1). Using PMA, superoxide generation was highly elevated and this effect was not increased by increasing extracellular calcium. When the neutrophils were preincubated in the presence of A23187, no effect of increasing the extracellular calcium concentration was observed on superoxide generation in PMA-activated neutrophils, whereas the cells became responsive to OAG in a highly calcium-dependent manner (Fig. 1).

Intracellular calcium and superoxide generation was measured in quin2-loaded neutrophils preincubated in the presence of ionomycin. When the cells were incubated at 2 mM Ca^{2+} with increasing ionomycin concentrations, and thus increasing

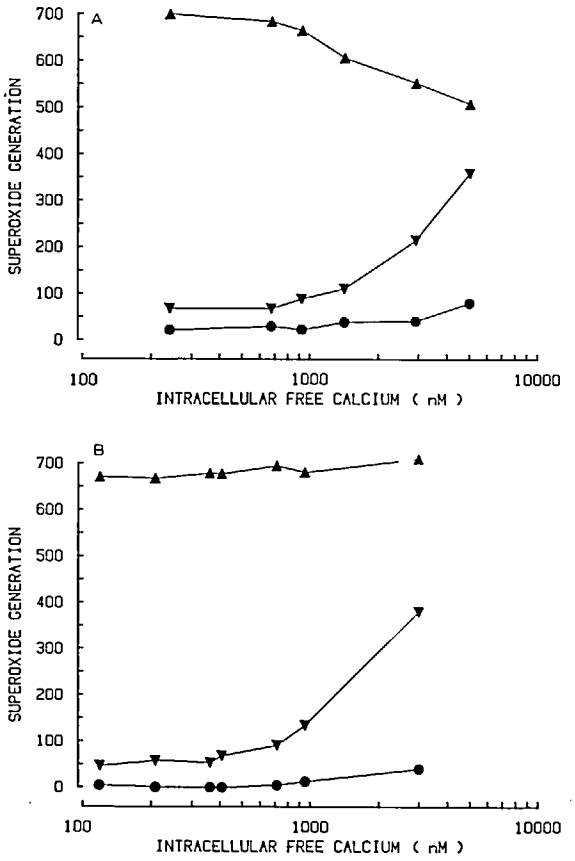

Fig. 2. Calcium activation of superoxide generation in OAG- and PMA-activated neutrophils prestimulated by ionomycin. $1 \cdot 10^7$ neutrophils/ml were isolated as in Ref. 11 and loaded with quin2 as described and preincubated for 10 min at 37°C either in buffer C (containing 2 mM $CaCl_2$) (A) or in buffer C with an increasing extracellular calcium concentration (B). The neutrophils were then activated for 5 min with increasing concentrations of ionomycin (A) or at constant ionomycin concentration (3 μM) (B). The intracellular calcium concentration was measured as described. Superoxide generation was measured as superoxide dismutase-inhibitable cytochrome *c* reduction and is expressed as the difference in absorbance at 550 nm ($\times 10^3$). ●, no addition but ionomycin; ▼, ionomycin plus 0.1 mg/ml OAG; ▲, ionomycin plus 1 μg/ml PMA.

concentrations of free intracellular calcium, a very small stimulation of the superoxide generation was observed at intracellular calcium concentrations above 1 μM (Fig. 2A). In the presence of PMA, superoxide generation was highly activated even at 0.25 μM calcium and did not increase further on increasing the intracellular calcium concentration. However, the effect of OAG on superoxide generation was highly potentiated by increasing the intracellular calcium concentration above 1 μM (Fig. 2A). Similar results were obtained using constant concentrations of ionomycin but varying extracellular calcium concentrations (Fig. 2B).

Activation of purified protein kinase C

Activation of purified protein kinase C was studied using quin2 as an indicator of the free calcium concentration in the mixture used for the assay. In the absence of phosphatidylserine, protein kinase C was not activated even at 2 mM Ca^{2+}. In the presence of phosphatidylserine, activation of the enzyme was only observed at calcium concentrations above 1 μM (Fig. 3). PMA or OAG in combination with phosphatidylserine induced a powerful activation of purified protein kinase C which was independent of calcium in the range 30–1000 nM. Even in the presence of EGTA and no added calcium, PMA and OAG activated purified protein kinase C. In the absence of phosphatidylserine, PMA or OAG did not activate protein kinase C.

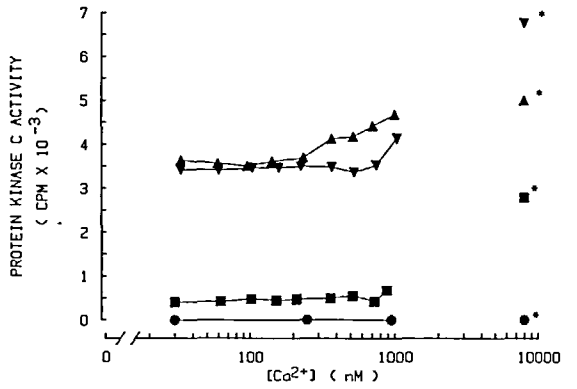

Fig. 3. Activation of purified protein kinase C. Protein kinase C activity was determined at different free calcium concentrations of the assay mixture in the presence of: (■) 40 μg/ml phosphatidylserine; (▼) 40 μg/ml phosphatidylserine, 0.1 mg/ml OAG (▲) 40 μg/ml phosphatidylserine, 1 μg/ml or PMA (●) unstimulated control. The symbols marked by an asterisk indicate maximal activity obtained in the presence of 2 mM calcium.

TABLE II

MEMBRANE-ASSOCIATED PROTEIN KINASE C ACTIVITY IN OAG-, FMLP- AND A23187-ACTIVATED NEUTROPHILS

Membrane-associated protein kinase C activity in $4 \cdot 10^7$ neutrophils was determined after stimulation for 5 min with FMLP, A23187, OAG or a combination of these activators. One representative experiment of three is shown.

Neutrophil stimulators	Particulate fraction associated protein kinase activity (mU/10^6 cells ± S.E., $n = 3$)
None	20 ± 3
FMLP (1 μM)	22 ± 2
OAG (0.1 mg/ml)	63 ± 11
FMLP (1 μM) and OAG (0.1 mg/ml)	62 ± 6
A23187 (2.5 μg/ml)	62 ± 2
A23187 (2.5 μg/ml) and OAG (0.1 mg/ml)	104 ± 2

Protein kinase C translocation in ionomycin-, A23187 and FMLP-stimulated neutrophils

FMLP did not induce a translocation of protein kinase C after 5 min stimulation (Table II).

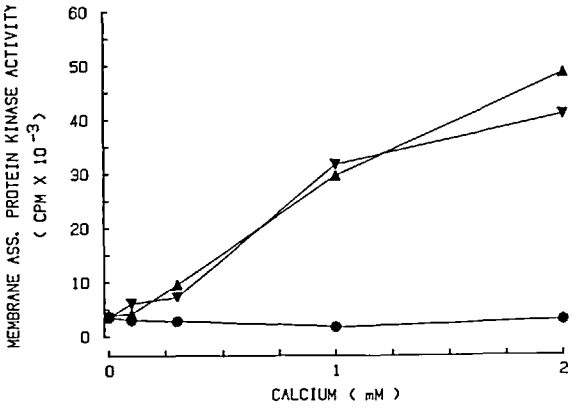

Fig. 4. Membrane-associated protein kinase C activity in A23187- and ionomycin-stimulated neutrophils at varying extracellular calcium concentrations. $4 \cdot 10^7$ neutrophils were isolated as in Ref. 12 and preincubated for 10 min at 37 °C in buffer A with calcium concentrations as indicated, and stimulated for 10 min with (▲) 3 μM ionomycin, (▼) 2.5 μg/ml A23187, or (●) unstimulated. Maximal membrane association was observed using 2 mM extracellular calcium.

When the leukocytes were stimulated with 1 μM FMLP for 0.5, 1, 2 and 4 min and immediately sonicated, no increase in membrane bound protein kinase C was observed (data not shown). The translocation induced by ionomycin and A23187 was strictly dependent upon the extracellular calcium concentration and no translocation was observed in the absence of extracellular calcium. Indeed, maximal translocation was first observed at 2 mM extracellular calcium (Fig. 4). In contrast, PMA- and OAG-induced translocation of protein kinase C was unaffected by extracellular calcium (data not shown).

Stimulation of $2 \cdot 10^7$ leukocytes/ml by 0.1 mg/ml OAG only induced partial translocation of protein kinase C, and in these submaximally OAG-stimulated cells, A23187 (Table II) and ionomycin (data not shown) enhanced the translocation. FMLP was unable to induce an elevation of membrane-associated protein kinase C activity in submaximally OAG-activated cells (Table II).

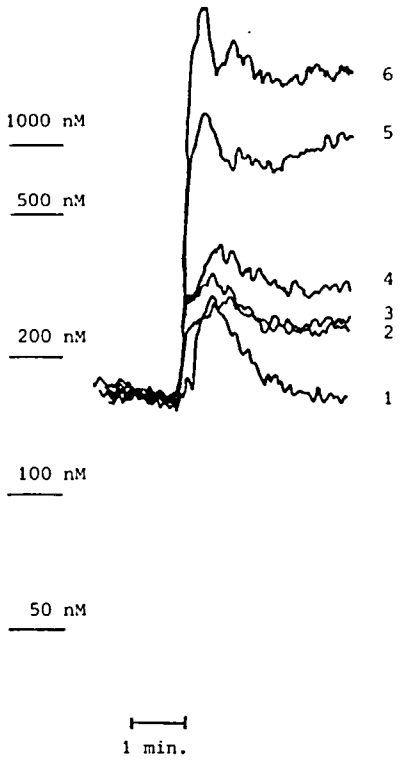

Fig. 5. Free intracellular calcium in ionomycin- and FMLP-stimulated neutrophils. Neutrophils were isolated as in Ref. 11 and loaded with quin2 as described, and the fluorescence was continuously measured after stimulation with FMLP or ionomycin: 1, 10^{-9} M FMLP; 2, 10^{-8} M FMLP; 3, 10^{-7} M FMLP; 4, 10^{-6} M FMLP; 5, 1 μM ionomycin; 6, 3 μM ionomycin.

Intracellular calcium in FMLP- and ionomycin-stimulated human polymorphonuclear leukocytes

FMLP activation of human polymoprhonuclear leukocytes elevated the intracellular calcium concentration to maximally 500 nM contrasting with ionomycin, which gave rise to maximal fluorescens of quin2 and thus increased intracellular calcium to at least 2 μM (Fig. 5).

Discussion

PMA and OAG activation of human polymorphonuclear leukocytes

Stimulation of protein kinase C has been hypothesized to induce activation of NADPH oxidase and thus superoxide formation. Both PMA and OAG have been assumed to exert their effects through an activation of protein kinase C [2,14]. However, as demonstrated in table I and in Ref. 15, PMA induces higher superoxide generation than OAG. One explanation, as proposed by Bonser et al. [15], is that either OAG is unable to penetrate the cell membrane or OAG is metabolized in the cell membrane. Therefore, superoxide generation and membrane-bound protein kinase C were measured in parallel experiments. The amount of translocated protein kinase C in OAG-stimulated cells was comparable to the PMA-induced translocation indicating that OAG equals PMA in its ability to stabilize complexes with protein kinase C in the cell membrane (Table I). In addition, at calcium concentrations corresponding to intracellular calcium, OAG was found to activate purified protein kinase C to the same extent as PMA (Fig. 3). An inhibitory effect of OAG upon superoxide generation could explain a lower effect of this diacylglycerol. However, superoxide generation in OAG-stimulated cells could be activated by ionomycin, indicating that OAG does not inhibit superoxide generation (Figs. 2A, 2B). Also in phorbol dibutyrate activated cells an inhibition by OAG could not be detected [15], and in PMA (1 μg/ml) -stimulated leukocytes no inhibition was observed by addition of OAG (0.1

mg/ml) (data not shown). Taken together, OAG (1) seems to stabilize complexes with protein kinase C in the cell membrane to the same extent as PMA, (2) seems to activate purified protein kinase C at calcium concentrations corresponding to those normally present intracellularly and (3) does not inhibit superoxide generation. Therefore, we conclude that although OAG and PMA differ in their ability to induce superoxide generation, protein kinase C seems to be equally activated in OAG- and PMA-stimulated neutrophils. This indicates that PMA induces some effects in addition to protein kinase C activation. Ozaki et al. [16] have demonstrated that OAG and PMA activation of superoxide generation differ in their dependency upon cytochalasin B. Also, the phosphorylation pattern in OAG and PMA activated HL-60 leukemia cells has been reported to differ and only PMA is able to induce maturation in these cells [17].

Influence of intracellular calcium upon superoxide generation in PMA- and OAG-activated leukocytes

A synergism between A23187 and OAG or PMA in superoxide production by human neutrophils has been reported [18,19]. Also, Ozaki et al. [16] have demonstrated partial dependency upon extracellular calcium in superoxide production by neutrophils stimulated by low concentrations of OAG, an effect which was eliminated using higher concentrations.

To investigate the role of intracellular calcium in PMA- and OAG-activated neutrophils, intracellular calcium was varied in two ways: (1) using constant extracellular calcium and increasing concentrations of ionophore and (2) using constant ionophore concentrations and increasing extracellular calcium. Both methods demonstrated that increasing the intracellular calcium concentration in OAG-stimulated neutrophils induced elevated superoxide generation. In ionomycin-stimulated leukocytes, intracellular calcium was further monitored using quin2 (Figs. 2A, B) and the effect of increasing intracellular calcium was only observed at calcium concentrations above 1 μM. Although the activity of OAG-stimulated protein kinase C is increased approx. 2-fold by elevating the calcium concentration from 1 μM to 2 mM (Fig. 3), this 2-fold increase is unlikely to explain the dramatic increase in superoxide generation observed upon elevation of intracellular calcium in OAG-stimulated neutrophils. Also, the synergism between ionophores and OAG cannot be explained by an increased membrane association of protein kinase C, since ionophores increased superoxide generation in maximally OAG-stimulated neutrophils without increasing membrane bound protein kinase C (data not shown).

In contrast to OAG, PMA activation of the neutrophils seemed to render the superoxide generation independent of intracellular calcium. Using quin2 as an intracellular calcium chelator PMA has been shown to activate superoxide generation at very low intracellular calcium concentrations [20].

Activation of human polymorphonuclear leukocytes by Ca^{2+} ionophores

Ionomycin and A23187 both induced increased protein kinase C activity in the particulate fraction (Fig. 4). In contrast to PMA and OAG, the ionophore-induced translocation was found to be strictly dependent upon extracellular calcium (data not shown). In fact, maximal translocation induced by the ionophore was first reached at 2 mM extracellular calcium indicating that the ionophore-induced translocation demands very high intracellular calcium concentrations.

As the ionophores induce translocation of protein kinase C and calcium elevation, it is likely that protein kinase C is activated in ionophore-stimulated leukocytes. However, compared to PMA and FMLP-activated cells, the superoxide production in ionophore-stimulated neutrophils is low (data not shown). The low superoxide generation in ionophore activated cells cannot be explained by inhibition of superoxide generation by ionophores, as PMA activation of superoxide generation was not inhibited by ionophores (Fig. 2A). Although both FMLP and ionophores are known to induce elevated intracellular calcium, only ionophores stimulate a tight membrane association of protein kinase C to the membrane fraction (Table II). As demonstrated in Fig. 5 and reported by Korchak et al. [21], calcium in FMLP-activated cells is only elevated to approx. 500 nM. This has to be compared to an elevation of more than 2 μM induced by ionophores. Therefore, the lack of

protein kinase C translocation in FMLP-stimulated cells may depend on differences in maximal calcium elevation. However, as both intracellular calcium and tight membrane-associated protein kinase C activity seem to be elevated to a greater extent by ionophores than by FMLP, the higher superoxide generation in FMLP-activated cells cannot solely be explained by either elevated intracellular calcium concentrations or tight membrane-associated protein kinase C, or these parameters in combination.

In conclusion, our results indicate that (1) although OAG and PMA differ in their ability to induce superoxide generation, protein kinase C seems to be equally activated in OAG- and PMA-stimulated neutrophils. (2) Neutrophils stimulated maximally by OAG increase superoxide generation upon elevation of intracellular calcium, and this is in contrast to maximally PMA-activated cells. (3) The increased superoxide generation induced by ionophores in OAG-stimulated neutrophils seems to be calcium dependent, but cannot solely be explained by an increased calcium activation of protein kinase C or an increased membrane-associated protein kinase C activity. (4) FMLP-induced superoxide generation cannot solely be explained by increased tight membrane-associated protein kinase C activity, elevated intracellular calcium or a combination of both. (5) Translocation of protein kinase C in PMA- or OAG-activated leukocytes occurs independently of extracellular calcium while ionophores induce this effect only in the presence of extracellular calcium.

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