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Ca^{2+}/Calmodulin-dependent Protein Kinase II Inhibitors Potentiate Superoxide Production in Polymorphonuclear Leukocytes

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Abstract

The possible role of Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II) in superoxide anion (O_2^-) production induced by formyl-methionyl-leucyl-phenylalanine (FMLP) was investigated in mouse polymorphonuclear leukocytes (PMNs).

KN-93 and KN-62, specific CaMK II inhibitors, augmented FMLP-induced O_2^- production. KN-92, an analogue which did not inhibit CaMK II, did not affect O_2^- production. W-7, a calmodulin inhibitor, augmented O_2^- production when administered at 30 mM for 5 min. KN-93 and recombinant mouse tumour necrosis factor-α (rmTNF-α) each augmented the maximal production of O_2^- induced by FMLP, and an additive effect of a combination of KN-93 and rmTNF-α was observed. CaMK II activity in the PMNs was increased by FMLP, and the increase was inhibited by KN-93 but not by rmTNF-α.

These results suggest that the inhibition of CaMK II resulted in the augmentation of FMLP-induced O_2^- production in PMNs by a mechanism different from that of the augmentation shown by TNF-α.

The stimulation of polymorphonuclear leukocytes (PMNs) by various stimuli evokes a rapid and massive production of superoxide anions (O_2^-), which play a pivotal role in host defence against microbial infection (Lehrer et al 1988). Depending on the extent of O_2^- production, this may also contribute to host tissue damage (Hogg 1987). Formyl-methionyl-leucyl-phenylalanine (FMLP) binds to cell surface receptors coupled to guanosine 5′-triphosphate (GTP) binding protein (Okajima & Ui 1984) and activates intracellular signal transduction pathways in PMNs, resulting in the activation of NADPH oxidase to generate O_2^-. Many enzymes such as phospholipase C, phospholipase D, phospholipase A_2, protein kinase C, phosphatidylinositol 3-kinase, MAP kinase, Ser/Thr kinase may be involved in these signal transduction pathways (Bokoch 1995). Since these multiple pathways appear to be quite complex, the regulation of O_2^- production remains to be elucidated. The suppressive regulation of O_2^- production, which may be important to protect the tissue from damage by massive amounts of O_2^-, is not yet understood.

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Cytokines such as tumour necrosis factor-α (TNF-α) do not activate O_2^- production by themselves but rather enhance it in response to soluble stimuli (Utsumi et al 1992). The mechanism(s) of this effect of TNF-α remain to be clarified. It is known that Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II) is an important enzyme in the intracellular Ca^{2+}-mediated signal transduction pathway of various tissues (Goldenring et al 1983; McGuinness et al 1985; Hanson & Schulman 1992). The involvement of CaMK II in the O_2^- production by PMNs is not yet known. In the present study, we found that CaMK II inhibitors potentiate FMLP-induced O_2^- production in a manner different from that shown by recombinant mouse TNF-α (rmTNF-α), suggesting a role of CaMK II in the suppressive regulation of O_2^- production in PMNs.

Materials and Methods

Materials

The following reagents were purchased: KN-93 {2–[*N*-2-(hydroxyethyl)-*N*-(4-methoxybenzenesulphonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzyl-

amine}, KN-92 {[2-*N*-(4-methoxybenzene-sulphonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methyl-benzylamine}, KN-62 {1-[*N*,*O*-bis(5-isoqunoline-sulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiper-azine} and W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide] from Seikagaku Co. (Tokyo, Japan); MCLA (2-methyl-6-(*p*-methox-yphenyl)-3,7-dihydroimidazo [1,2-α] pyrazin-3-one hydrochloride) and oyster glycogen from Tokyo Kasei (Tokyo, Japan); FMLP from Sigma Chemical Co. (St Louis, MO); rmTNF-α from Boehringer Mannheim (Mannheim, Germany); Autocamtide-3 from Life Technologies, Inc. (Gaithersburg, MD); [γ-^{32}P]ATP from Du Pont NEN (Boston, MA); Dextran 200 000 and the other reagents from Wako Pure Chemical Industries (Osaka, Japan).

Preparations of leukocytes

Blood was collected by cardiac puncture from 5-week-old male ddY strain mice, weighing 25–30 g, obtained from Japan SLC (Shizuoka, Japan). Leukocytes were separated by dextran sedimentation followed by the hypotonic lysis of remaining erythrocytes. The leukocytes were suspended in Hanks' Balanced Salt Solution (HBSS (mM): 138 NaCl, 5·36 KCl, 0·34 Na_2HPO_4, 0·44 KH_2PO_4, 0·41 $MgSO_4$, 0·49 $MgCl_2$, 1·26 $CaCl_2$, 4·0 $NaHCO_3$, 5·55 glucose, pH 7·4) at a concentration of 10^6 cells mL^{-1} (mouse leukocytes). The PMNs used in certain experiments were harvested from mice 15 h after an intraperitoneal injection of 0·5 mL of 0·3% oyster glycogen. The PMNs were suspended in HBSS at a concentration of 5×10^5 cells mL^{-1} (mouse PMNs). The ratios of PMNs in the mouse leukocytes and mouse PMNs were 25% and 90%, respectively.

Measurement of O_2^-

The production of O_2^- was determined by MCLA-dependent chemiluminescence using a luminescence reader (BLR-301, Aloka, Tokyo) as described by Nakano et al (1986). Mouse leukocytes (10^6 cells) or mouse PMNs ($2{\cdot}5 \times 10^5$ cells) in 2 mL HBSS were incubated at 37°C in a CO_2 incubator for 10 min, and MCLA (0·5 mM) was then added. After 15 min, the background and the stimulus-evoked chemiluminescence was measured every 5 s for 3 min. The O_2^- production was expressed as integrated counts for 3 min. Each value represented the average ± s.e.m. of 4–6 preparations. Unless otherwise described, KN-93 and rmTNF-α were added at 5 min and 25 min before the stimulations, respectively. The FMLP-induced chemiluminescence was completely abolished by the addition of 75 U superoxide dismutase (Wako, Osaka).

Assay of CaMK II activity

CaMK II activity was measured as described by Fukunaga (1989) with slight modifications. Briefly, leukocytes (4×10^5 cells) were incubated for 25 min with 0·6 mM okadaic acid and then stimulated with 1 mM FMLP. After 1 min, the reaction was terminated by liquid nitrogen. KN-93 was added 5 min before the addition of FMLP. Cells were lysed by sonication in 50 mM HEPES (pH 7·4) containing 0·1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM $Na_4P_2O_7$, 100 mM β-glycerophosphate, 25 mM NaF, 0·1 mM leupeptin, 75 μM pepstatin A and 0·1 mg mL^{-1} aprotinin. Cell lysates were incubated in 50 mM HEPES (pH 7·4) containing 60 μM [γ-^{32}P]ATP (1000 counts min^{-1} pmol^{-1}), 1 μM EGTA, 10 mM $MgCl_2$, 0·01% Tween 20, and 0·1 mM H-89 with or without 60 μM Autocamtide-3 for 10 min at room temperature to determine the Ca^{2+}/calmodulin independent protein kinase activities. The reaction was stopped by the addition of trichloroacetic acid. After centrifugation at 2000 *g* for 3 min, samples of the supernatant were then spotted on P81 phosphocellulose papers. The papers were washed, dried, and counted as described elsewhere (Roskoski 1983). The difference between the radioactivity of samples with and without Autocamtide-3 was calculated as the enzyme activity.

Results

In mouse leukocytes, FMLP (1 μM) induced a rapid and transient production of O_2^- which reached a peak at 25–30 s and disappeared completely 2 min after the stimulation (Figure 1A). When mouse leukocytes were first incubated with 10 μM KN-93, an inhibitor of CaMK II (Sumi et al 1991; Niki et al 1993), for 5–25 min, the FMLP-induced production of O_2^- was markedly augmented; the maximal effect was obtained by preincubation for 5 min (Table 1). The augmentation by preincubation for 5 min was dependent on the concentrations of KN-93 over the range 1-10 μM (Figure 1A). The O_2^- production was increased by KN-93 (10 μM, 5 min), to 363 ± 6·9% (n = 6) chemiluminescence for 3 min in mouse leukocytes, in which lymphocytes coexisted at a high rate. However, the same augmentations (248 ± 16·5%, n = 6) were observed in mouse PMNs. We have preliminary data suggesting that the O_2^- production was augmented to the same degree by KN-93 (10 μM, 5 min) in PMNs separated from human peripheral blood, in which lymphocytes were negligible. These results showed

that the effect of KN-93 on PMNs was direct and not indirect via coexisting lymphocytes. Since the treatment did not affect the O_2^- production induced by zymosan and phorbol 12-myristate 13-acetate, which continued over 30 min, in these preparations (data not shown), the action of KN-93 seemed to affect specifically the rapid and transient O_2^- production induced by FMLP.

To further test whether the augmentation of O_2^- production by KN-93 was due to the inhibition of CaMK II, the effects of related compounds on FMLP-induced O_2^- production were also tested (Table 1). KN-62, an alternative CaMK II inhibitor (Tokumitsu et al 1990), augmented O_2^- production to 155% and 166% by treatment for 5 min and 25 min, respectively. KN-92, a structural analogue of KN-93 which does not inhibit CaMK II (Tombes et al 1995), had no effect on O_2^- production. W-7, an inhibitor of Ca^{2+}/calmodulin-dependent enzymes (Hidaka et al 1981), augmented O_2^- production by two-fold only by treatment for 5 min at 30 μM. The augmentation was not observed in the longer treatment, and the treatment at 100 μM for 25 min rather suppressed O_2^- production (control: 58 717 ± 5952 counts/3 min; W-7: 14 139 ± 8779). The treatment with a high concentration of W-7 may have suppressed the calmodulin action necessary for O_2^- production. Therefore, these results suggest that an augmentation of FMLP-induced O_2^- production may be elicited by CaMK II inhibition.

rmTNF-α, in contrast, enhanced the FMLP-induced O_2^- production only when administered for longer than 10 min (Table 1), and the maximal effect was obtained by 7·5 ng mL^{-1} for 25 min, to 249 ± 10·4% (n = 6) in mouse leukocytes (Figure 1B) and to 220 ± 21·7% (n = 6) in mouse PMNs. Neither KN-93 nor rmTNF-α affected the MCLA-dependent chemiluminescence evoked by hypoxanthine/xanthine oxidase (data not shown), suggesting that KN-93 and rmTNF-α had no effect on the MCLA-dependent chemiluminescence induced by O_2^-.

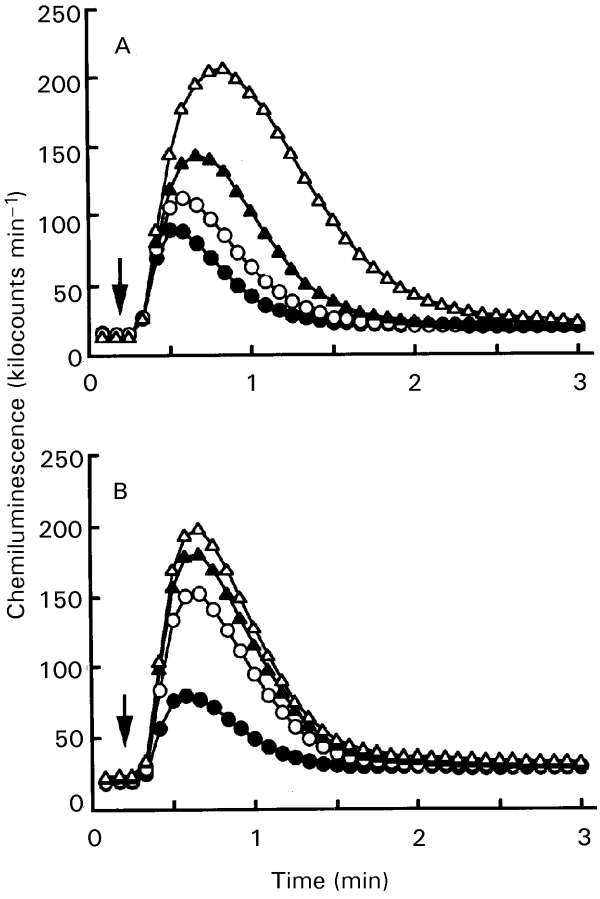

Figure 1. Time course of FMLP-induced O_2^- production augmented by KN-93 (A) and rmTNF-α (B) in mouse leukocytes. Mouse leukocytes (10^6 cells/2 mL) treated without (●) and with KN-93 (1 μM: ○; 3 μM: ▲; and 10 μM: △) for 5 min or with rmTNF-α (0·75 ng mL^{-1}: ○; 2·5 ng mL^{-1}: ▲; and 7·5 ng mL^{-1}: △) for 25 min were stimulated with 1 μM FMLP as indicated by arrows, and the chemiluminescence was measured. Each symbol represents the mean of six preparations.

The O_2^- production was increased by FMLP in a concentration-dependent manner over the range 0·01–1 μM and reached a plateau at 1–10 μM. KN-93 (10 μM) and rmTNF-α (7·5 ng mL^{-1}) markedly augmented the O_2^- production induced by 0·1–10 μM FMLP (Figure 2). It is noteworthy that the

Table 1. Effects of inhibitors and rmTNF-α on FMLP-induced O_2^- production by mouse leukocytes

Pretreatment (n)	control	5 min	25 min
KN-93 (4)	42,458 ± 5,385	153,668 ± 17,577***	109,834 ± 6,664***
KN-62 (5)	48,422 ± 2,554	75,048 ± 2,659**	80,407 ± 7,886**
KN-92 (4)	47,469 ± 3,522	45,429 ± 2,659	41,502 ± 2,659
W-7 (4)	44,225 ± 4,196	99,722 ± 11,669**	45,052 ± 5,272
rmTNF-α (4)	52,982 ± 5,599	60,071 ± 5,002	127,750 ± 8,779***

Leukocytes were preincubated with KN-93 (10 μM), KN-62 (10 μM), KN-92 (10 μM), W-7 (30 μM) or rmTNF-α (7·5 ng mL^{-1}) for 5 min or 25 min, and the chemiluminescence induced by 1 μM FMLP was measured. Each value represents the mean ± s.e.m. chemiluminescence value (counts/3 min) of the number of leukocyte preparations indicated in parentheses. ***$P < 0·001$, **$P < 0·01$ vs. control.

maximal production induced by 1–10 μM FMLP alone was markedly augmented by KN-93 and rmTNF-α. However, the augmentation by each was somewhat different. By the treatment with KN-93, the peak time after the stimulation was delayed (control: 25 s; KN-93 1 μM: 30 s, 3 μM: 30 s, 10 μM: 40 s) with a slight change in the initial rate of O_2^- production (control: $356 \pm 32{\cdot}8$ kilocounts min^{-2}; KN-93 1 μM: $501 \pm 33{\cdot}4$, 3 μM: $565 \pm 57{\cdot}0$, 10 μM: $569 \pm 37{\cdot}6$) (Figure 1A). rmTNF-α increased the initial rate (control: $375 \pm 31{\cdot}0$ kilocounts min^{-2}; rmTNF-α 0·75 ng mL^{-1}: $661 \pm 76{\cdot}6$, 2·5 ng mL^{-1}: $787 \pm 96{\cdot}7$, 7·5 ng mL^{-1}: $834 \pm 83{\cdot}0$) without changing the peak time (control: 25 s; 0·75 ng mL: 30 s, 2·5 ng mL^{-1}: 30 s, 7·5 ng mL^{-1}: 30 s) (Figure 1B). Mouse leukocytes were then treated simultaneously with KN-93 and rmTNF-α at the concentrations of each which induced the maximal augmentation. Namely, both rmTNF-α (7·5 ng mL^{-1}) and KN-93 (10 μM) were administered 25 min or 5 min before the stimulation by FMLP. As depicted in Figure 3, the combination induced a greater augmentation of O_2^- production than that induced by either compound alone. The profile of O_2^- production by the combination was shifted to that of rmTNF-α as the initial rates were 257 ± 62 kcounts min^{-2}(control), 396 ± 10 (KN-93), 609 ± 49 (rmTNF-α), and 717 ± 24 (the combination), and the peak times were 25 s (control), 40 (KN-93), 30 (rmTNF-α), and 40 (the combination). The effect of the combination of KN-93 and rmTNF-α seems to be additive, suggesting that the mechanisms of the augmentation by each are different.

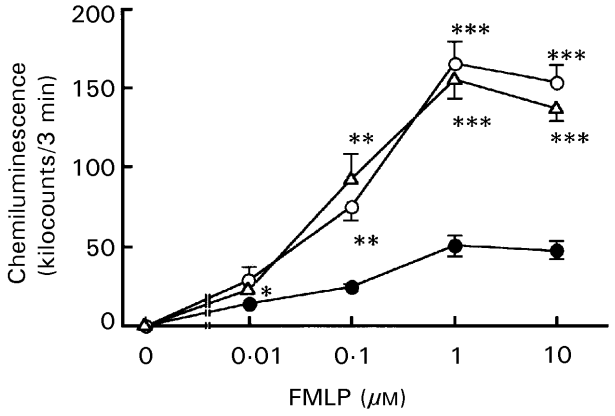

Figure 2. Effects of KN-93 and rmTNF-α on the dose–response curve of FMLP-induced O_2^- production in mouse leukocytes. The experiment was carried out as described in the legend for Figure 1 except that the stimulation was by 10 nM–10 μM FMLP. Each symbol plus bar represents the mean ± s.e.m. of six preparations. Control ●, KN-93 (10 μM) ○, rmTNF-α (7·5 ng mL^{-1}) △. $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$ vs control.

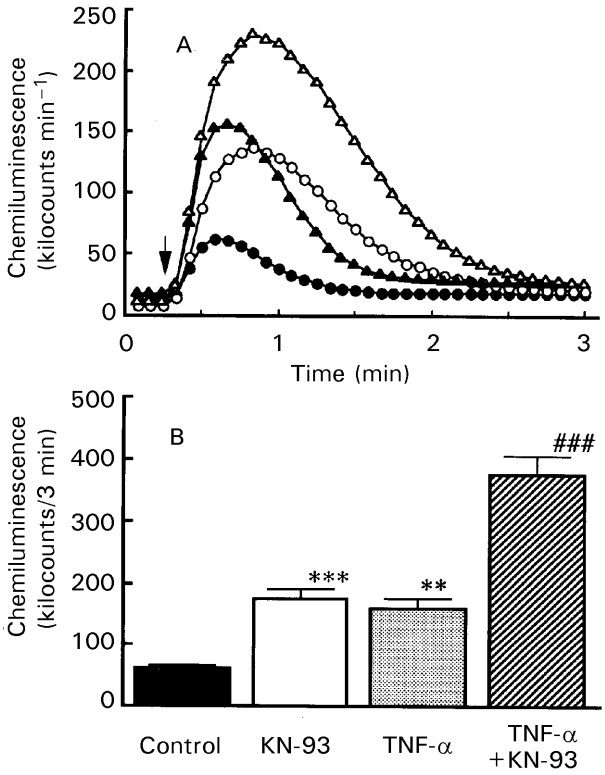

Figure 3. Augmentation of FMLP-induced O_2^- production by a combination of KN-93 and rmTNF-α in mouse leukocytes. The experiment was carried out as described in the legend for Figure 1 except that leukocytes were dually treated with KN-93 (10 μM) for 5 min and rmTNF-α (7·5 ng mL^{-1}) for 25 min in one group. A. Time course of O_2^- production. Control ●, KN-93 ○, rmTNF-α ▲, KN-93 + rmTNF-α: △. Each symbol represents the mean of six preparations. B. Chemiluminescence for 3 min. Each column plus bar represents the mean ± s.e.m. of six preparations. $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$ vs control; $^{###}P < 0{\cdot}001$ vs KN-93.

The involvement of CaMK II in O_2^- production has not yet been clarified. Here, FMLP increased the CaMK II activity to 210% in mouse leukocytes (Figure 4) and to 140% in mouse PMN (data not shown). This increase was completely inhibited by 10 μM KN-93 (Figure 4), but not by rmTNF-α (data not shown). These results suggest that CaMK II is involved in O_2^- production.

Discussion

The specific cross-linking of Fc γ-receptor type II in human neutrophils induces CaMK II activation and O_2^- production (Liang & Huang 1995). However an involvement of CaMK II in the signalling responses of respiratory burst by PMNs has not been reported. In the present study, the O_2^- production induced by FMLP was markedly augmented by KN-93 and KN-62, which are specific inhibitors of CaMK II but not of protein kinase A,

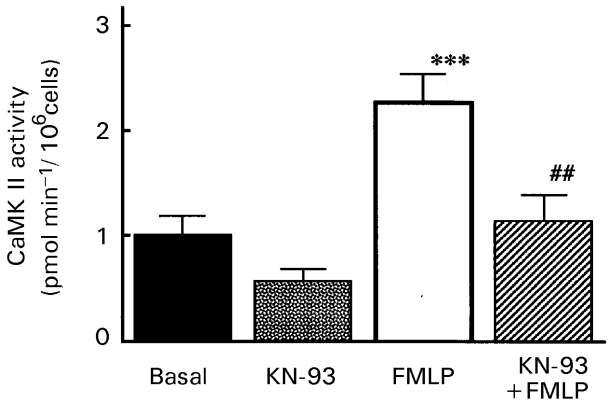

Figure 4. Effect of KN-93 on CaMK II activity in mouse leukocytes. Mouse leukocytes were incubated with 0·6 μM okadaic acid for 25 min and stimulated with 1 μM FMLP. After 1 min, leukocytes were frozen in liquid nitrogen and the CaMK II activity was evaluated as described in Methods and Materials. KN-93 (10 μM) was added 5 min before the stimulation by FMLP. Each column plus bar represents the mean ± s.e.m. of six preparations. ***$P < 0.001$ vs basal, ##$P < 0.01$ vs FMLP.

myosin light chain kinase, protein kinase C or casein kinase I (Tokumitsu et al 1990; Sumi et al 1991). KN-62 has been shown to strongly inhibit CaMK IV (Enslen et al 1994), but KN-93 does not (Ishida et al 1995). KN-62 is a hydrophobic isoquinolinesulphonamide derivative (Tokumitsu et al 1990), while KN-93 is a hydrophilic methoxybenzensulphonamide (Sumi et al 1991; Niki et al 1993). Although their chemical characters are completely different, both KN-62 and KN-93 have been shown to specifically inhibit the autophosphorylation of both the α- and β-subunits of CaMK II, in a competitive fashion against calmodulin (Tokumitsu et al 1990; Sumi et al 1991). These inhibitors have been found, on detailed study, to have undesirable non-specific actions (Mamiya et al 1993; Marley & Thompson 1996), which complicate their use in certain types of experiments. However, such complications were excluded here by comparing the actions of inhibitors with that of KN-92, an inactive analogue, which did not affect the FMLP-induced O_2^- production. W-7, an inhibitor of Ca^{2+}/calmodulin-dependent enzymes, augmented the production only by treatment for 5 min at 30 μM; higher concentrations of W-7 rather inhibited O_2^- production in a similar manner to the observations made with human neutrophils reported by Perianin et al (1994). Since FMLP-induced O_2^- production is dependent on an increase in intracellular Ca^{2+}, pretreatment with a high concentration of W-7 for a longer time might induce a defect of intracellular Ca^{2+} action. The cellular modifications by W-7 may affect distinct calmodulin-dependent events that may have a different role in the mechanism of O_2^- production. We suspect that under certain conditions W-7 might inhibit CaMK II without markedly affecting these distinct calmodulin-dependent events. It was verified here that CaMK II was activated by FMLP and that the activation was inhibited by KN-93 in mouse PMNs. Therefore, the actions of KN-93, KN-62 and W-7 clearly suggested that the inhibition of CaMK II elicited the augmentation of FMLP-induced O_2^- production.

TNF-α is known to enhance FMLP-induced O_2^- production in PMNs. The mechanism of the priming action of TNF-α has been shown to be due to tyrosyl phosphorylation (Akimaru et al 1992), an increase of GTP-binding protein in the cell membrane (Klein et al 1995), the upregulation of chemoattractant receptors (O'Flaherty et al 1991), the activation of phospholipase A (DiPersio et al 1988) and an increase in intracellular Ca^{2+} (Schumann et al 1993). However, the entire mechanism of TNF-α action remains to be determined. In the present study, it was suggested that CaMK II inhibitors augmented the FMLP-induced O_2^- production by mechanism(s) different from those reported for rmTNF-α.

The present study was carried out using suspensions of PMNs and the action of CaMK II inhibitors was observed specifically on rapid and transient O_2^- production. The respiratory burst of PMNs in suspension differs in major respects from that of PMNs adherent to the extracellular matrix. Adherent PMNs exhibit a lag period of several minutes before the onset of FMLP-induced oxidant production, with a maximal response observed at 60–120 min (Nathan 1987). The oxidant release is linked to the tissue injury that accompanies a variety of pathologic processes (Henson & Johnston 1987). In contrast, PMNs in suspension exhibit a transient production of O_2^- within 1 min in response to FMLP and O_2^- production is terminated after 2–3 min. Thus a mechanism for quickly terminating O_2^- production might be involved in the suspension. An alternative explanation is that an increase in intracellular Ca^{2+} induced by FMLP activates both stimulatory and suppressive regulations of O_2^- production. The removal of the suppressive regulation should result in an increase in maximal O_2^- production and a delay in the time course of FMLP-induced O_2^- production. This speculation is supported by the present finding as follows. Firstly, in the present study, CaMK II activity was not affected by CaMK inhibitors alone, but FMLP-induced increase in CaMK II activity was inhibited by the inhibitors. Secondly, CaMK II inhibitors alone did not induce O_2^- production but augmented the maximal O_2^- production by FMLP

alone. Thirdly, CaMK II inhibitors delayed the time required for the O_2^- production to reach a peak and to terminate, implying a removal of the suppressive regulation. We suspect that CaMK II is involved in the suppressive regulation of O_2^- production and CaMK II inhibitors might remove the suppressive regulation.

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