

# Phospholipase C and phosphatidylinositol 3-kinase signaling are involved in the exogenous arachidonic acid-stimulated respiratory burst in human neutrophils

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**Abstract:** To define the role of phospholipase C (PLC) and phosphatidylinositol 3-kinase (PI-3K), signaling pathways in arachidonic acid (AA)-stimulated respiratory burst in human neutrophils, the AA-stimulated respiratory burst, Ins(1,4,5)P<sub>3</sub> production, PI-3K activation, and cytoplasmic Ca<sup>2+</sup> mobilization were investigated. It was found that Ins(1,4,5)P<sub>3</sub> production and PI-3K activity in AA-stimulated cells were increased in a dose-dependent manner. U73122, the PLC inhibitor, effectively inhibited the AA-stimulated respiratory burst and Ca<sup>2+</sup> release from the intracellular calcium store but not the activity of PI-3K, indicating the independence of PI-3K signaling on PLC activation. Wortmannin, the PI-3K inhibitor, at the concentration sufficient to inhibit PI-3K activity, can only partially inhibit Ca<sup>2+</sup> release from the internal store, indicating a partial regulation of PLC signaling by PI-3K and the existence of two pathways initiated by different PLC subfamilies. One is regulated by PI-3K activation, and the other is independent of PI-3K signaling. It was observed that AA could still induce a noncapacitative Ca<sup>2+</sup> entry in the cells when Ca<sup>2+</sup> release from the intracellular store was blocked by a PLC inhibitor, or a capacitative Ca<sup>2+</sup> entry was induced by preincubation with thapsigargin. However, the AA-mediated, noncapacitative Ca<sup>2+</sup> entry seems to play a little, if any, role in the stimulated respiratory burst. The present study suggests that the PLC signaling pathway, which may be activated by PLC<sub>β</sub> and PLC<sub>γ</sub>, respectively, and the PI-3K signaling pathway are involved in the AA-stimulated respiratory burst in human neutrophil. *J. Leukoc. Biol.* 74: 428–437; 2003.

**Key Words:** signal transduction · calcium mobilization · calcium entry · U73122 · wortmannin

## INTRODUCTION

The production of superoxide radical, O<sub>2</sub><sup>-</sup>, by polymorphonuclear neutrophils in the ischaemia-reperfused tissue has been known as an important pathological factor of the injury [1]. Possibly mediated by some chemotactic factors [2, 3], the

neutrophils cross the endothelial membrane of the vascular bed into the ischemic myocardium [4, 5] and then may be activated by some stimulating substance formed under an ischemic condition to release O<sub>2</sub><sup>-</sup> and lysosomal enzymes. Besides the presence of complement fragments, which gains access to the myocardial extravascular space by crossing the endothelial membrane of the vascular tree [4], arachidonic acid (AA) accumulates in the ischemic myocardium [6] as the second potential activating agent for neutrophils [7]. Understanding the mechanism of neutrophil activation by exogenous AA will certainly be helpful for seeking measures to prevent or reduce the ischemia-reperfusion injury.

Intracellular AA is released from membrane phospholipids by two main pathways: cleavage of the glycerophospholipid at the sn-2 position by various forms of activated phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and alternatively, hydrolysis of diacylglycerol (DAG) catalyzed by two DAG-lipase [8]. In addition to serving as the precursor to a plethora of eicosanoids and other bioactive molecules, AA may play a role as a second messenger in regulating many cell functions or as a stimulator to activate other cells when it released into the extracellular space. As previously reported, AA can directly activate the reduced nicotinamide adenine dinucleotide phosphate oxidase [9] and protein kinase C (PKC) [10] in vitro system, stimulate translocation of PKC to a particulate fraction in the effects of AA on mitogen-activated protein kinase, such as p38 and c-Jun N-terminal kinase and phosphorylation [11, 12], and activate a guanosine 5'-triphosphate (GTP)-binding protein in the neutrophil plasma membrane by increasing the number of available GTP-binding sites [13]. There were also some studies showing that endogenous AA activates plasma membrane Ca<sup>2+</sup> channels, promoting Ca<sup>2+</sup> entry from the extracellular space [14]. However, the mechanisms, through which the exogenous AA stimulates neutrophil leukocytes to undergo respiratory burst, have not been thoroughly studied.

In comparison with the studies on the mechanisms involved in the respiratory burst stimulated by chemoattractants and cytokines, the studies on the respiratory burst stimulated by exogenous AA in neutrophils were rather limited. Until Hii

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and his coworkers [15] reported that AA stimulated the activity of class Ia phosphatidylinositol 3-kinase (PI-3K) in human umbilical vein endothelial cells, HL60 cells, and human neutrophils, only a few published papers had dealt with the signal pathways involved in the exogenous AA-stimulated respiratory burst in neutrophils. In those papers, some  $\text{Ca}^{2+}$ -binding proteins, including calmodulin [16, 17], GTP-binding protein [18], activation of  $\text{PLA}_2$  [19], leukotriene  $\text{B}_4$  ( $\text{LTB}_4$ ) receptor, and the leukotriene synthesis through an  $\text{LTB}_4$ -stimulated autocrine loop [20], were suggested to be involved. However, no investigation about the involvement of the phospholipase C (PLC) signaling, which is one of most important signaling pathways in the chemoattractant-stimulated respiratory burst in neutrophils, has been reported in AA-stimulated respiratory burst. For this reason, direct measurement of inositol trisphosphate ( $\text{IP}_3$ ) production in AA-stimulated neutrophils was made to know whether the PLC pathway plays any role in the AA-stimulated respiratory burst in the present study.

It was known quite recently that AA can induce  $\text{Ca}^{2+}$  entry or cytoplasmic  $\text{Ca}^{2+}$  mobilization, which completely differs from the capacitative or store-operated mode for receptor-activated  $\text{Ca}^{2+}$  entry in human embryo kidney (HEK293) cells [21–23]. Does the so-called “noncapacitative”  $\text{Ca}^{2+}$  entry exist in the AA-stimulated neutrophils? Does AA induce a cytoplasmic calcium mobilization through the  $\text{Ca}^{2+}$  entry regulated by depletion of the internal calcium stores? To answer these questions is also a goal for the present investigation.

As the  $\text{Ca}^{2+}$  release from intracellular calcium stores is triggered by binding of the  $\text{IP}_3$  receptor to  $\text{IP}_3$ , the product of PLC activation, to determine the role of the  $\text{Ca}^{2+}$  release from internal stores in the AA-stimulated respiratory burst may also provide a better justification about the possible involvement of PLC signaling.

Although the activation of PI-3K in AA-stimulated neutrophils has been reported [15], the cross-talk between PLC and PI-3K signaling pathways in the AA-stimulated respiratory burst has not been well defined. Thus, the effect of U73122 (1-[6-((17 $\beta$ )-3-methoxyoestra-1,3,5(10)-trien-17-yl)-amino-hexyl]-1H-pyrrole-2,5-dione), the inhibitor of PLC [24], on the activity of PI-3K or the effect of wortmannin, the selective inhibitor of PI-3K [25], on PLC-dependent  $\text{Ca}^{2+}$  release from intracellular calcium stores were investigated. It has been reported that the growth factor-induced PLC activation was enhanced by activation of PI-3K in nonphagocytes [26, 27], and the zymosan- or bacteria-induced phosphorylation of  $\text{PLC}_\gamma$  was inhibited by a PI-3K inhibitor in mouse macrophages [28]; nevertheless, it is still unclear to what extent the PLC signaling depends on the activation of PI-3K in AA-stimulated neutrophils.

## MATERIALS AND METHODS

### Chemicals

AA, EGTA, Dextran T-500 (molecular weight = 500,000), phosphatidylinositol 4-monophosphate [ $\text{PI}(4)\text{P}$ ], phosphatidylinositol 4,5-diphosphate [ $\text{PI}(4,5)\text{P}_2$ ], U73122 (1-[6-((17 $\beta$ )-3-methoxyoestra-1,3,5(10)-trien-17-yl)-amino-hexyl]-1H-pyrrole-2,5-dione), and U73343 (1-[6-((17 $\beta$ )-3-methoxyoestra-1,3,5(10)-trien-17-yl)-amino-hexyl-pyrrole-dione) were purchased from Sigma Chemical

Co. (St. Louis, MO). 1,2-Bis(*O*-aminophenyl-ethane-ethane)-*N,N,N',N'*-tetraacetic acid-acetoxymethyl ester (BAPTA/AM) and Fura-2/AM were from Molecular Probes (Junction City, OR). Lymphocyte-separating solution (density:  $1.007 \pm 0.002$ ) was purchased from the Institute of Hematology, Chinese Academy of Medical Sciences (Tianjing). The D-myo- $\text{IP}_3$  [ $^3\text{H}$ ] assay system was purchased from Amersham Pharmacia Biotech (Little Chalfont, UK). [ $\gamma$ - $^{32}\text{P}$ ]Adenosine 5'-triphosphate (ATP) was from Yan-Huai Co. (Beijing, China). Other reagents are all of analytical grade. Stock solution of U73122, BAPTA/AM, and Fura-2/AM (1 mM) was prepared in dimethyl sulfoxide (DMSO). When it was added in cell suspension, the final concentration of DMSO never exceeded 0.1%. AA was first prepared in DMSO at 500 mM and was then diluted to 1 mM by acidic distilled water (pH 3.0) to avoid oxidation.

### Cell preparation

Human neutrophils were isolated from heparinized healthy donor blood, according to the following procedure. Blood was mixed with 4.5% Dextran in 0.9% NaCl solution. The red cells were settled for 40 min at 4°C, and leukocyte-rich plasma was layered on the top of the lymphocytes separating solution in sterilized tubes. The neutrophils were obtained as a pellet after centrifugation at 500 *g* for 15 min. Contaminating erythrocytes were removed by hypotonic lysis. The cells were finally washed twice with saline and suspended in Hank's balanced salt solution (HBSS) containing 1.3 mM  $\text{CaCl}_2$  (pH 7.4) at the concentration of  $10^6$  cells/ml. Except for the sedimentation of red cells, all other isolation procedures were performed at room temperature. The viability of neutrophils was checked by Trypan blue exclusion and was always found to be greater than 95%. The purity of neutrophils was checked by Wright's staining and was found to be higher than 98%.

### Chemiluminescence measurement of respiratory burst

The respiratory burst of neutrophils was measured by a chemiluminescence method in which the emitted light from the reaction of luminol with the superoxide radical ( $\text{O}_2^-$ ) and  $\text{H}_2\text{O}_2$  generated by cells was detected as a concomitant chemiluminescence burst. Each 2 ml neutrophil suspension ( $10^6$  cells/ml) containing 0.1  $\mu\text{g}$  luminol was added in a quartz cuvette. Two identical cuvettes containing testing or controlled cell suspension were placed in a rotatable sample holder of a laboratory-made photon counter and measured at 37°C. As the respiratory burst of the cells for study and the respiratory burst of the controlled cells were measured simultaneously, the errors introduced by the time-dependent variation in cell vitality were minimized to a great extent.

### Measurement of cytoplasmic calcium mobilization using Fura-2

Neutrophils ( $10^6$  cells/ml) were loaded with Fura-2 by incubation with 1  $\mu\text{M}$  Fura-2/AM at 37°C for 45 min. Thereafter, the cells were washed twice to remove the extracellular dye and resuspended in HBSS at a density of  $10^6$  cells/ml. The loaded neutrophils were transferred in a magnetic stirring cuvette and were measured at 37°C on a dual excitation fluorescence spectrophotometer (Hitachi F-4500), which allows simultaneous excitation at 340 nm and 380 nm. During the measurement, AA was injected into the cell suspension. The fluorescence of the cytoplasmic  $\text{Ca}^{2+}$ -binding Fura-2 excited at 340 nm and 380 nm,  $F_{340}$  and  $F_{380}$ , was simultaneously recorded at the emission of 510 nm. The free cytoplasmic calcium concentration,  $[\text{Ca}^{2+}]_i$ , in cells was calculated according to the following equation [29]:  $[\text{Ca}^{2+}]_i = K_d [(R - R_{\min}) / (R_{\max} - R)] (F_{380, \min} / F_{380, \max})$ , where  $K_d$  is the dissociation constant for  $\text{Ca}^{2+}$  and Fura-2 (224 nM);  $R = F_{340} / F_{380}$ ;  $R_{\max} = F_{340, \max} / F_{380, \max}$ ;  $R_{\min} = F_{340, \min} / F_{380, \min}$ ;  $F_{340, \max}$  and  $F_{380, \max}$  are the fluorescence excited at 340 nm and 380 nm, respectively, at a saturated  $\text{Ca}^{2+}$  concentration, achieved by addition of 2  $\mu\text{l}$  Triton in 2 ml cell suspension.  $F_{340, \min}$  and  $F_{380, \min}$  are the fluorescence excited at 340 and 380 nm, respectively, at zero  $\text{Ca}^{2+}$  concentration, achieved by following addition of 2 mM EGTA in the tritonized cell suspension. When cytoplasmic calcium mobilization was measured in the cells suspended in  $\text{Ca}^{2+}$ -free buffer, the ratio of  $F_{340} / F_{380}$  was directly used to characterize the calcium mobilization.

### Measurement of PI-3K activity

The PI-3K activity in AA-stimulated cells was assayed by measuring the incorporation of the  $^{32}\text{P}$ -phosphate into  $\text{PI}(3,4)\text{P}_2$  in reference to literature [30].

31]. Briefly, 100 s later after stimulation, the cells were rapidly cooled down in an ice bath. After centrifugation, the cells were lysed with 500  $\mu$ l lysis buffer (0.25 M sucrose, 20 mM HEPES, 1 mM EGTA, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml antipain, 10  $\mu$ g/ml pepstatin A, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin) following further sonication. The cell lysate was then centrifuged at 100,000  $g$  for 30 min at 4°C; PI-3K was obtained as the supernatant and stored at 4°C for PI-3K activity assay. An aliquot (100  $\mu$ l) of the supernatant was mixed with [ $\gamma$ - $^{32}$ P]ATP (final concentration of 100  $\mu$ M and 5  $\mu$ Ci assay) and 200  $\mu$ g/ml sonicated PI(4)P at 30°C for 10 min. The reaction was stopped by addition of 100  $\mu$ l 1 N HCl and 200  $\mu$ l chloroform/methanol (2:1, v/v). Then, after shaking, the organic layer was collected, washed twice with 200  $\mu$ l 1 N HCl/methanol (1:1, v/v), and finally analyzed by thin-layer chromatography (TLC) on a silica gel plate (TLC aluminum sheets, Merck KgaA, Germany). The gel plates were developed in a mixture of chloroform, methanol, 25% ammonium hydroxide, and water (70:100:15:25, v/v). The PI(3,4)P<sub>2</sub> bands on the TLC sheet were identified using PI(4,5)P<sub>2</sub> as the marker, and the radioactivities of the  $^{32}$ P incorporated in the PI(3,4)P<sub>2</sub> bands were measured by the Storm 820 imaging system (Amersham Pharmacia Biotech), using storage Phosphor technology. All assays were performed in duplicate.

### Assay of Ins(1,4,5)P<sub>3</sub> production

The  $^3$ H-IP<sub>3</sub> competitive-binding assay [32] using the IP<sub>3</sub> [ $^3$ H] assay system quantitated the generation of IP<sub>3</sub> in neutrophils after AA stimulation. Neutrophils ( $4 \times 10^6$ ) in 4 ml HBSS buffer were stimulated with AA for 60 s at 37°C and then cooled down rapidly in an ice bath. The cells were harvested by centrifugation and lysated with 400  $\mu$ l ice-cold 4% perchloric acid. The acid-insoluble component was sedimented by centrifugation at 2000  $g$  for 15 min at 4°C. The supernatant was neutralized to pH 7.5 by ice-cold 10 M KOH. KClO<sub>4</sub> in the neutralized mixture was removed again by centrifugation at 4°C. An aliquot of each neutralized supernatant (100  $\mu$ l) was then used to quantitate the IP<sub>3</sub> concentration according to the protocol provided by the manufacturer.

## RESULTS

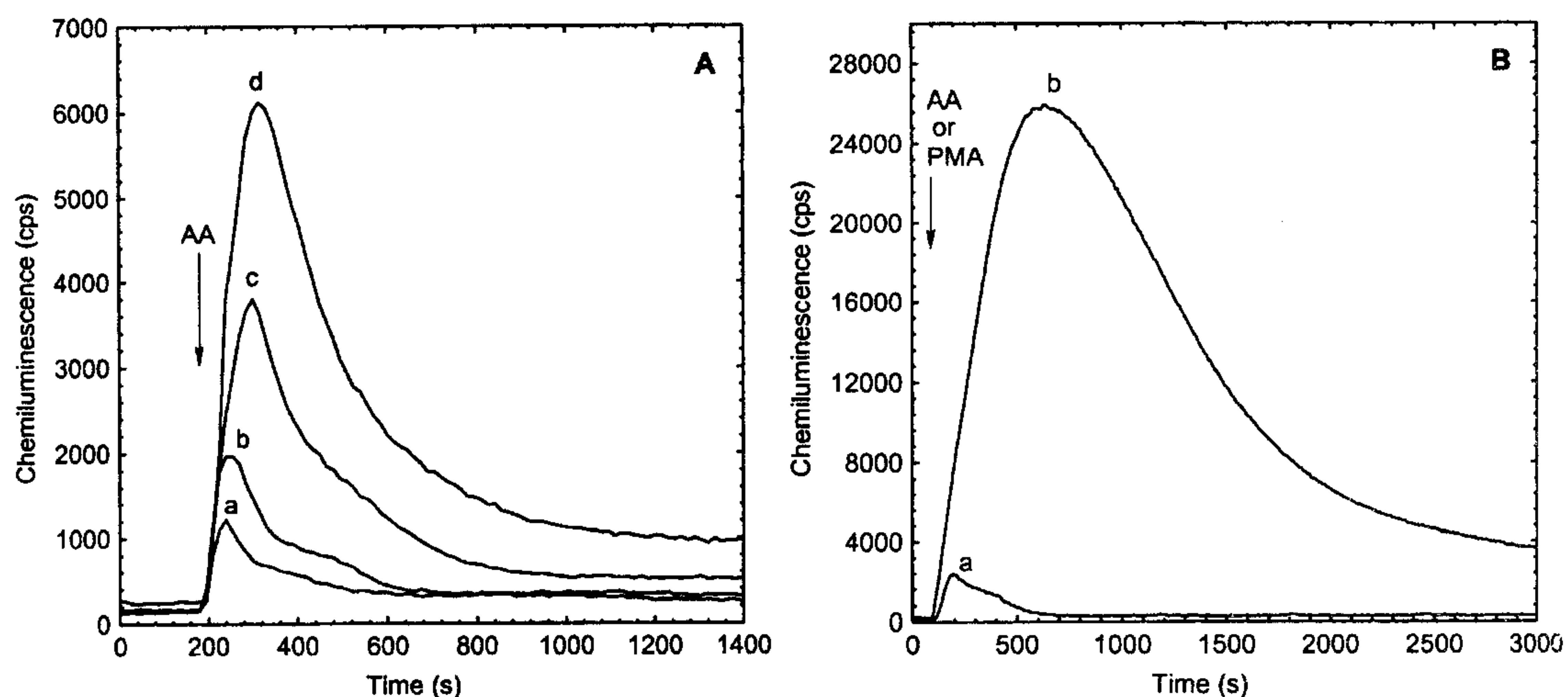
### Dependence of the respiratory burst and the intracellular Ca<sup>2+</sup> mobilization on AA concentration

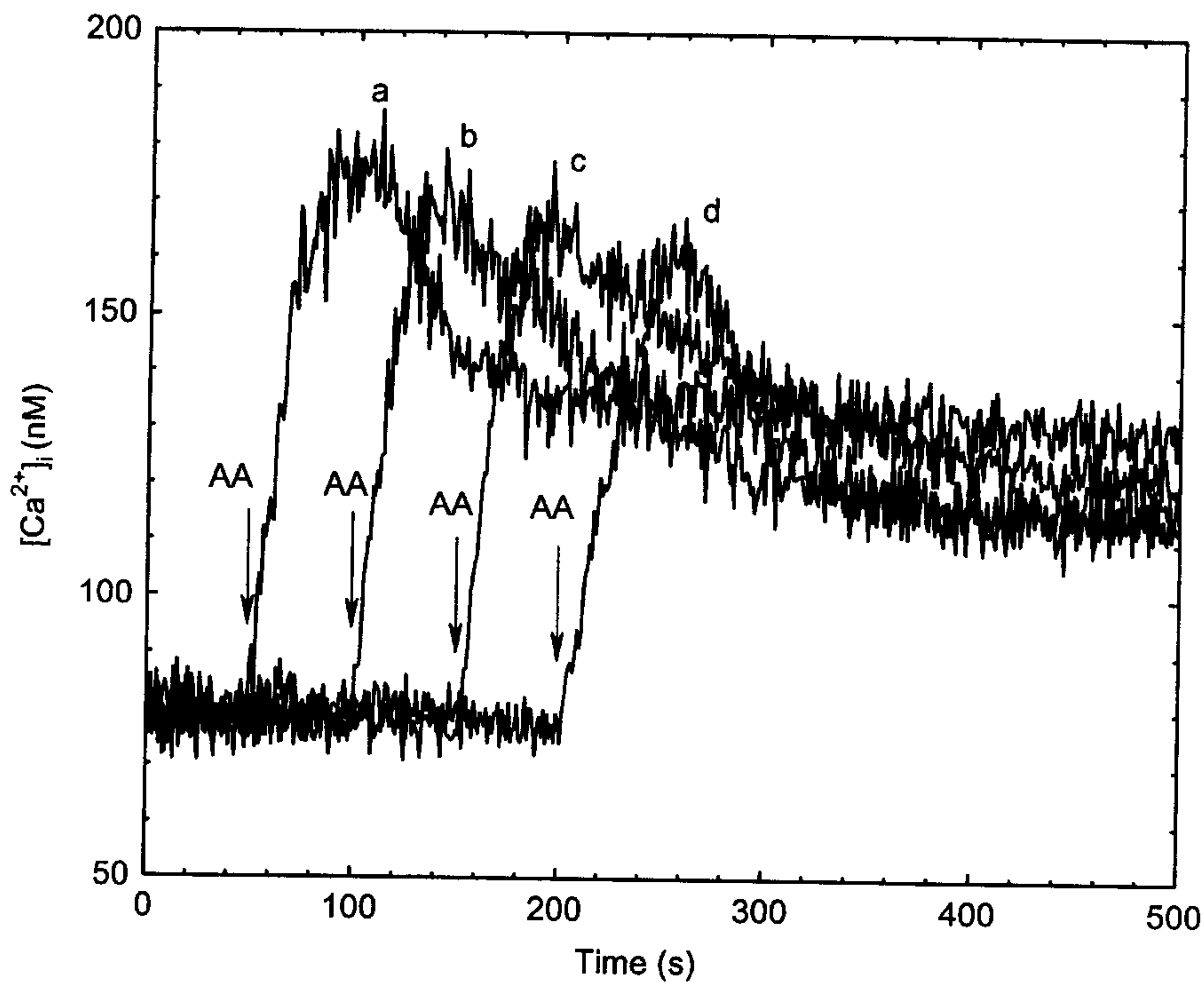
As the first glance of the AA-induced respiratory burst in neutrophils, various concentrations of AA were rapidly added in cell suspension, and the stimulated respiratory burst was kinetically recorded as the concomitant chemiluminescence burst. As shown in **Figure 1A**, as the concentration of AA increased from 2.5  $\mu$ M to 10  $\mu$ M, the intensity of the respiratory burst in neutrophils increased about sixfold. Although the

kinetics of the AA-induced respiratory burst varied to some extent from one cell preparation to another, the respiratory burst often reached its maximum between 40 and 120 s after the addition of AA, and the whole process was completed within 10–20 min. To know how sensitively the human neutrophil responds to AA stimulation and how dynamically the respiratory burst of neutrophils can be measured by the chemiluminescence method, the respiratory burst of the cells stimulated by 5  $\mu$ M AA and 100 nM PMA was measured, respectively. As shown in **Figure 1B**, although AA-stimulated respiratory burst concomitant chemiluminescence was rather weak in comparison with that stimulated by PMA, the chemiluminescence method was still sensitive enough to detect the AA-stimulated respiratory burst in neutrophils. In addition, it is evident that the AA-stimulated respiratory burst is much faster than the burst stimulated by PMA. Accordingly, the intracellular-free calcium mobilization induced by AA at various concentrations from 2.5  $\mu$ M to 10  $\mu$ M was also measured. The results are shown in **Figure 2**. It can be seen that a transient rise of intracellular-free calcium is induced in the neutrophils by addition of AA in cell suspension. The calcium mobilization exhibits a fast-rising phase and a relatively slow phase in declining; although the dose dependence of the AA-induced calcium mobilization is less than that observed in the AA-stimulated respiratory burst, the induced calcium mobilization still increases with increasing concentration of AA. A separate measurement showed that the intracellular Ca<sup>2+</sup> was raised from 100 nM to 200 nM in the cells stimulated by 5  $\mu$ M AA and from 100 nM to 280 nM in the cells stimulated by 20  $\mu$ M AA.

The ischemia and reperfusion experiments with rat heart showed that the AA content in heart tissue was approximately 20 nmol/g dry weight and then increased to ~80 and 120 nmol/g dry weight after 45 min ischemia and subsequent 45 min reperfusion, respectively [33]. It means that the AA concentration in the heart tissue of rat may be 16–24  $\mu$ M after ischemia and subsequent reperfusion. Based on the literature's data and the consideration that AA at high concentration may change the permeability of the plasma membrane of cells, a lower concentration of 5  $\mu$ M, which is close to the physiological condition, was chosen to study the signal pathways in-

**Fig. 1.** The respiratory bursts stimulated by various concentrations of AA and phorbol myristate acetate (PMA) in human neutrophils. (A) Cells ( $10^6$  cells/ml) were suspended in the Ca<sup>2+</sup> containing HBSS and were then stimulated by (a) 2.5  $\mu$ M AA, (b) 5  $\mu$ M AA, (c) 7.5  $\mu$ M AA, and (d) 10  $\mu$ M AA at 37°C, respectively. (B) Cells ( $10^6$  cells/ml) were suspended in the Ca<sup>2+</sup> containing HBSS and were then stimulated by (a) 5  $\mu$ M AA and (b) 100 nM PMA, respectively. The burst was measured as concomitant chemiluminescence. cps, Counts per second.



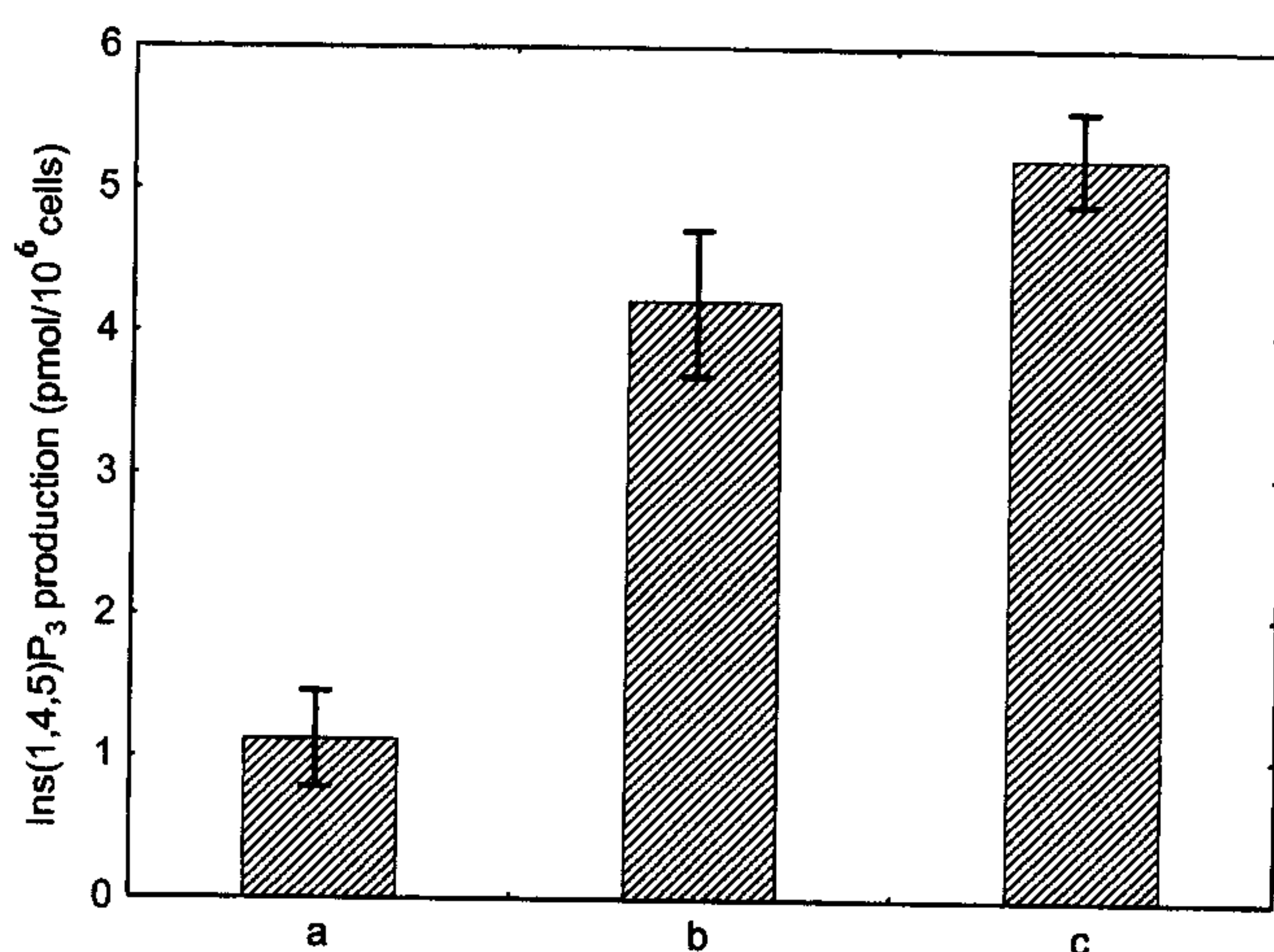


**Fig. 2.** The cytoplasmic calcium mobilization induced by various concentrations of AA in the Fura-2-loaded human neutrophils. The cells ( $10^6$  cells/ml) were suspended in the  $\text{Ca}^{2+}$  containing HBSS and were then stimulated by (a)  $10 \mu\text{M}$  AA, (b)  $7.5 \mu\text{M}$  AA, (c)  $5 \mu\text{M}$  AA, and (d)  $2.5 \mu\text{M}$  AA at  $37^\circ\text{C}$ . The loading of Fura-2 and the measurement are described in Materials and Methods.

involved in the AA-stimulated respiratory burst and the AA-induced calcium mobilization throughout the investigation.

### Dose-dependent production of $\text{Ins}(1,4,5)\text{P}_3$ in AA-stimulated cells

To know if the PLC activation is involved in AA-stimulated respiratory burst, direct measurement of its activity in AA-stimulated neutrophils seems absolutely necessary. For this reason, the production of  $\text{IP}_3$  in AA-stimulated cells was measured by competitive binding of the generated  $\text{IP}_3$  of cell extract to the  $\text{IP}_3$ -binding proteins against a constant amount of  $^3\text{H}$ -labeled  $\text{IP}_3$ . The results are shown in **Figure 3**. It was



**Fig. 3.** Production of  $\text{Ins}(1,4,5)\text{P}_3$  in resting and AA-stimulated neutrophils. The competitive binding with  $^3\text{H}$ -labeled  $\text{IP}_3$  was used to assay the  $\text{IP}_3$  production in the cells ( $10^6$  cells/ml) without stimulation (column a) and the cells stimulated by  $5 \mu\text{M}$  (column b) and  $10 \mu\text{M}$  (column c) AA, respectively. Each data is the mean of two independent measurements, and the SD is indicated as the bar.

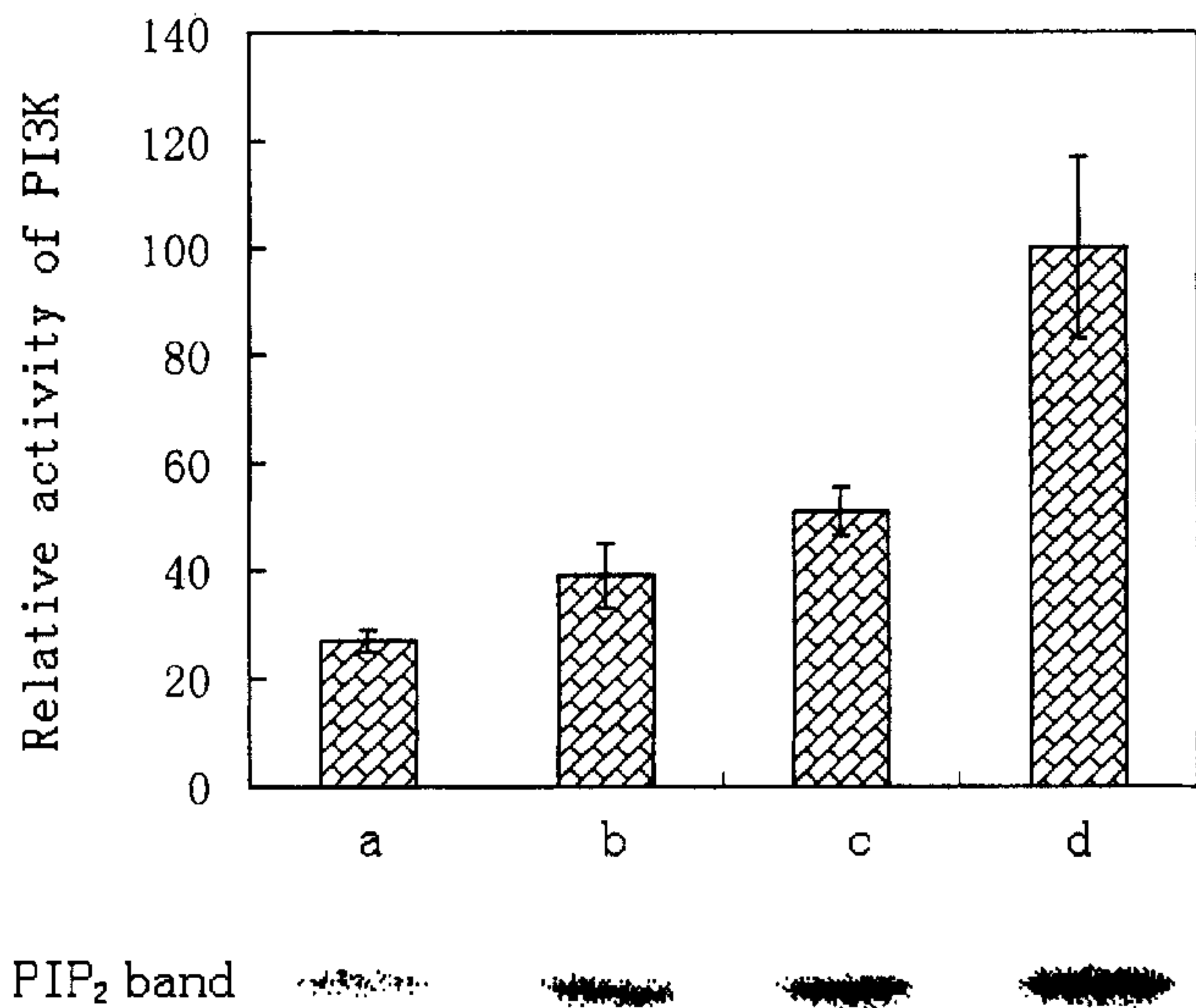
found that  $5 \mu\text{M}$  AA stimulation increased the  $\text{IP}_3$  production from  $(1.1 \pm 0.4) \times 10^{-18}$  moles per cell in resting cells to  $(4.2 \pm 0.6) \times 10^{-18}$  moles per cell in the stimulated neutrophils. The  $\text{IP}_3$  production in the cells stimulated by  $10 \mu\text{M}$  AA was further increased to  $(5.2 \pm 0.5) \times 10^{-18}$  moles per cell. The results indicate that AA activated the PLC in neutrophils.

### Dose-dependent activation of PI-3K by AA

To verify the involvement of PI-3K activation in AA-stimulated neutrophils, the activity of PI-3K was determined after AA had stimulated the cells at various concentrations for 100 s. As shown in **Figure 4**, the PI-3K activity in the cells stimulated by  $2.5 \mu\text{M}$ ,  $5.0 \mu\text{M}$ , or  $10 \mu\text{M}$  AA is  $\sim 1.6$ -, two-, and fourfold of that in the resting cells, respectively. The results shown in **Figures 1A** and **4** may suggest that the AA-stimulated respiratory burst increased with increasing activity of PI-3K.

### The effect of wortmannin on the AA-stimulated respiratory burst

To further prove the dependence of the AA-stimulated respiratory burst on the activity of PI-3K in neutrophils, wortmannin, a selective inhibitor of PI-3K, was used to treat the cells and to see its effect on the stimulated respiratory burst and the stimulated activity of PI-3K. The respiratory burst and the PI-3K activities in  $5 \mu\text{M}$  AA-stimulated neutrophils, suspended in HBSS containing 0, 10, 40, and 100 nM wortmannin, were assayed, respectively. The results are shown in **Figures 5** and **6**. It can be seen that as the concentration of wortmannin increased, the respiratory burst and PI-3K activity decreased. However, some activity of PI-3K already existed in the quiescent cells. It was noticed that 100 nM wortmannin inhibited the respiratory burst completely but only inhibited 65% of the PI-3K activity in AA-stimulated cells. The existing PI-3K activity in quiescent cells and remaining PI-3K activity

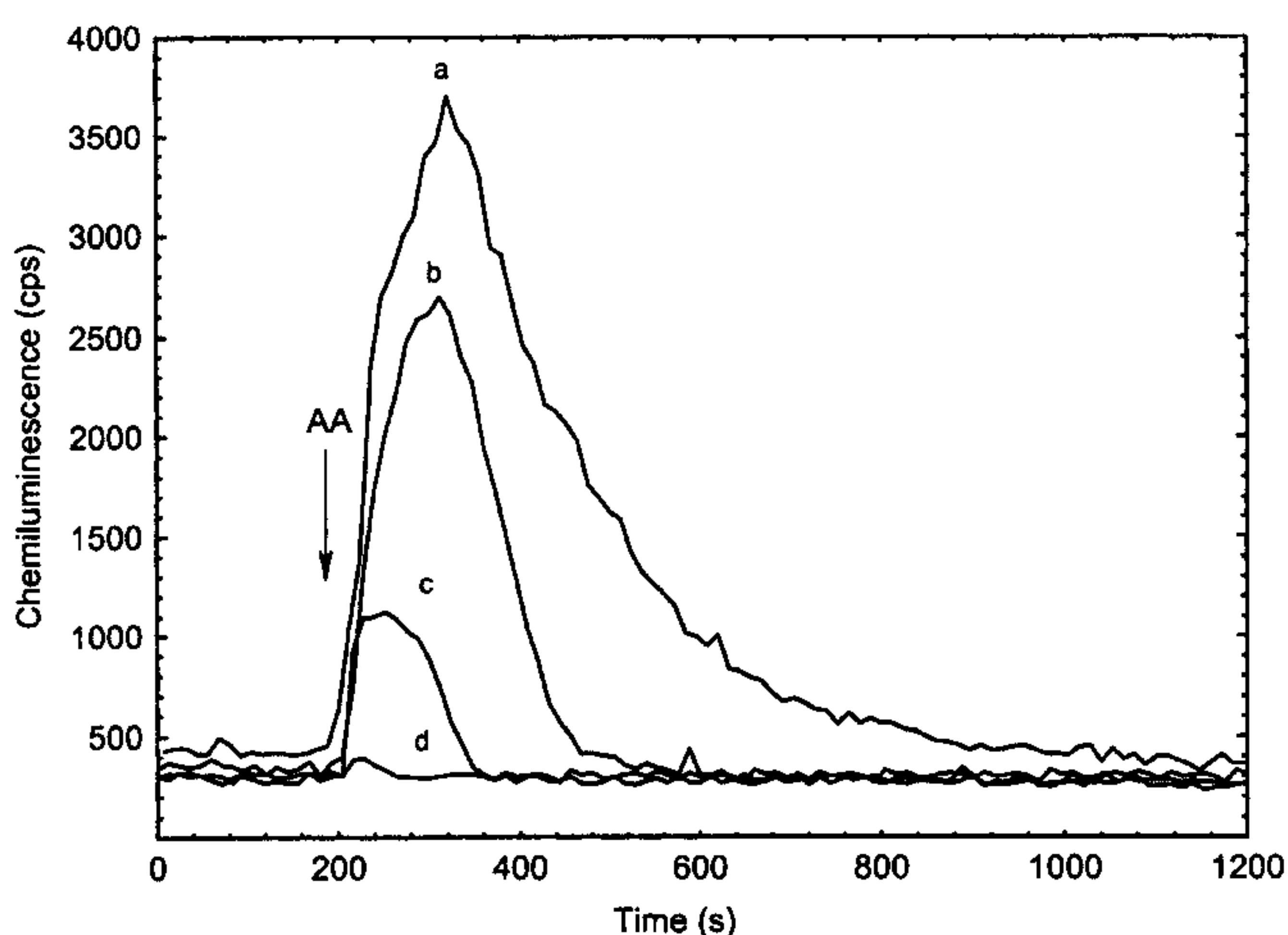


**Fig. 4.** Relative PI-3K activities in the neutrophils stimulated by various concentrations of AA. The columns and the bands of  $^{32}\text{P}$ -labeled PI(4,5) $\text{P}_2$  ( $\text{PIP}_2$ ) correspond to (a) 0  $\mu\text{M}$  AA, (b) 2.5  $\mu\text{M}$  AA, (c) 5  $\mu\text{M}$  AA, and (d) 10  $\mu\text{M}$  AA stimulation, respectively. Each data is the mean of three independent measurements, and the SD is indicated as the bar.

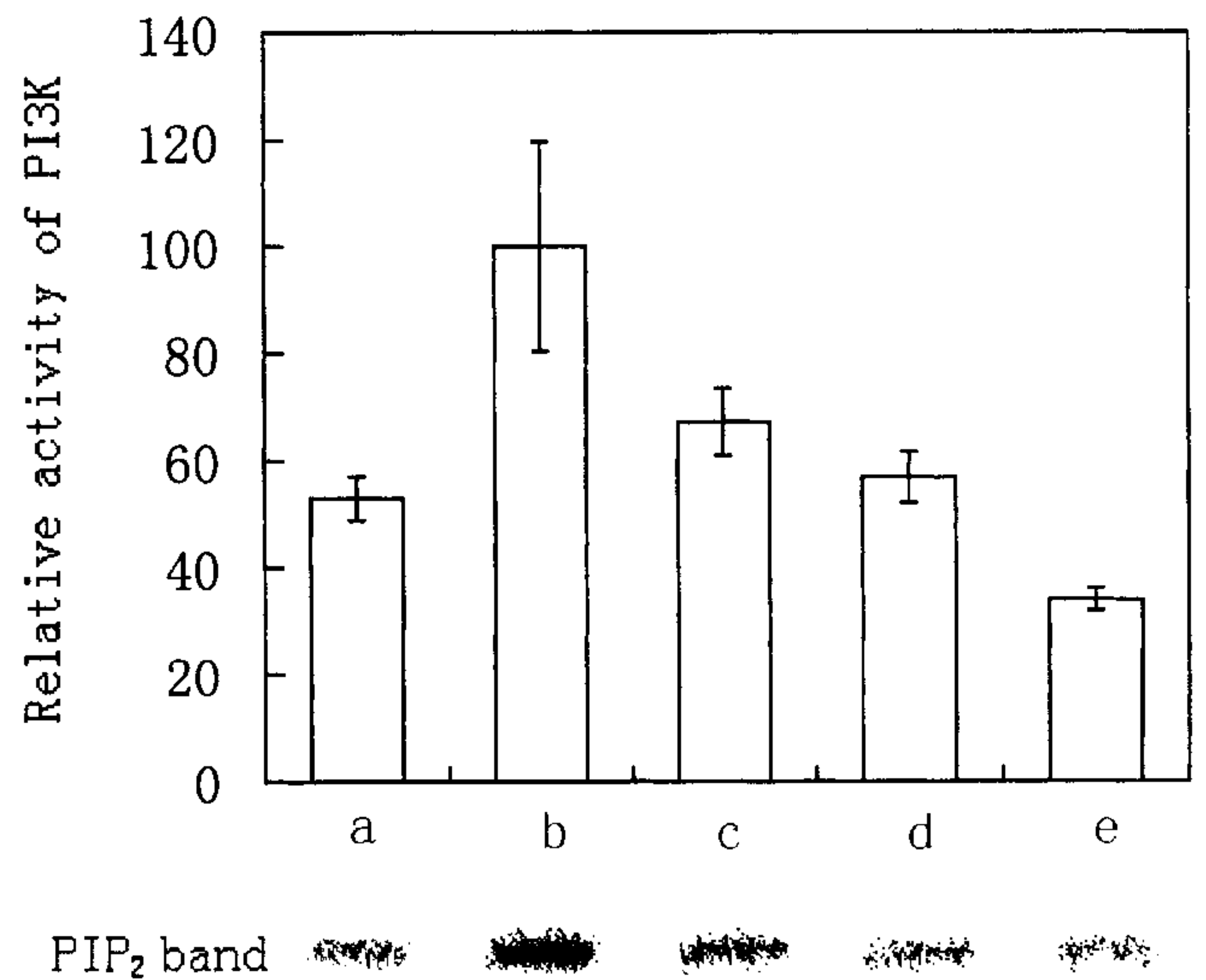
in the AA-stimulated cells, whose respiratory burst is completely inhibited by wortmannin, imply that there may be a threshold level of PI-3K activity in cells. Only when PI-3K activity exceeds this threshold can the neutrophil undergo respiratory burst.

#### Role of extracellular and intracellular $\text{Ca}^{2+}$ in the AA-induced respiratory burst

As calcium is an important second messenger in the signal transduction involved in the neutrophils activated by many stimuli such as chemoattractants and cytokines, it is natural to know whether the calcium plays a role in the AA-stimulated

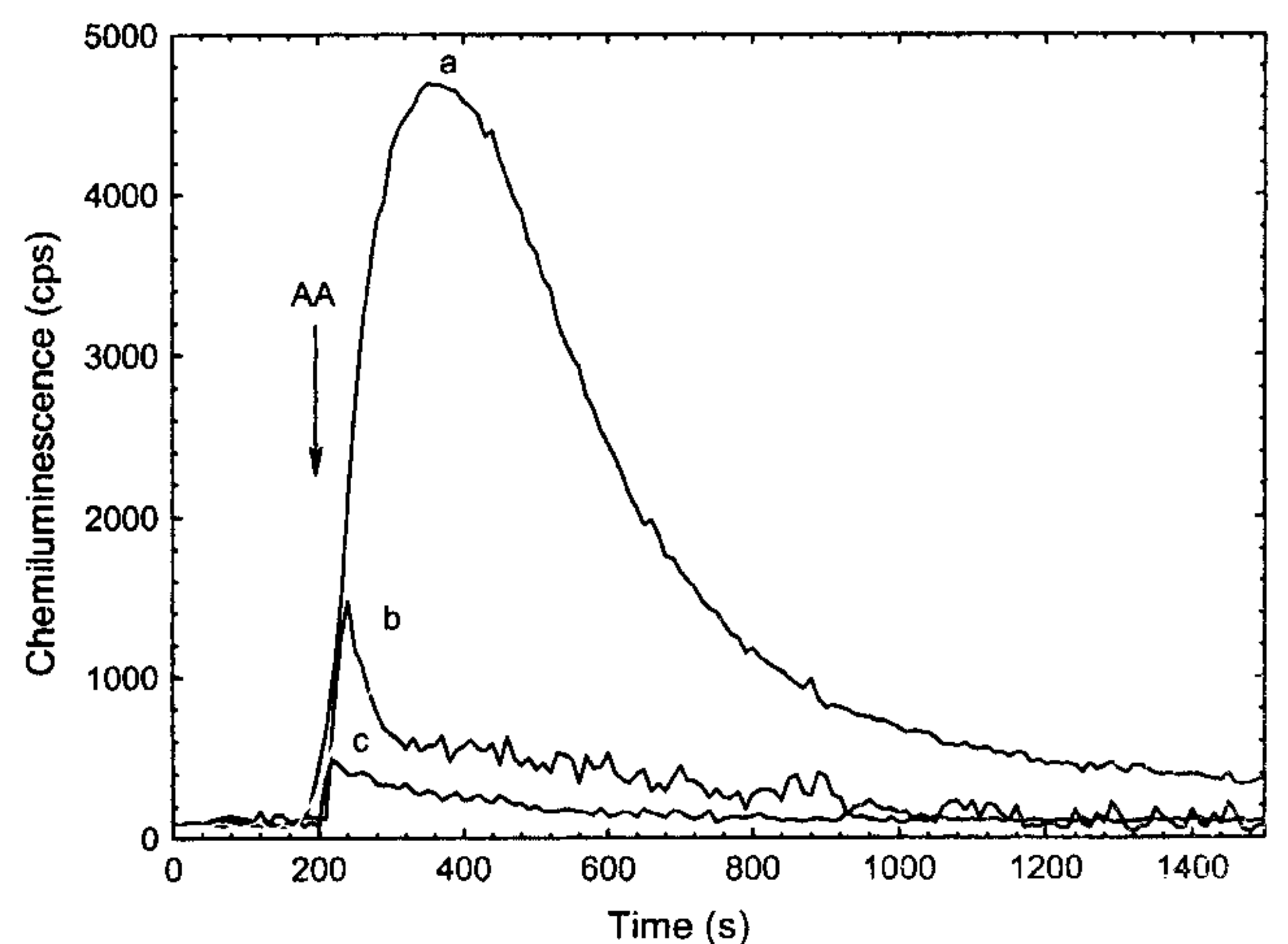


**Fig. 5.** The inhibitory effect of wortmannin on AA-stimulated respiratory burst in human neutrophils. The cells ( $10^6$  cells/ml; HBSS containing 0.1  $\mu\text{g}$  luminol, pH 7.2) were preincubated with wortmannin at 0 nM (a), 10 nM (b), 40 nM (c), and 100 nM (d), respectively, for 10 min and were then stimulated with 5  $\mu\text{M}$  AA at 37°C. The respiratory burst was measured as the concomitant chemiluminescence.

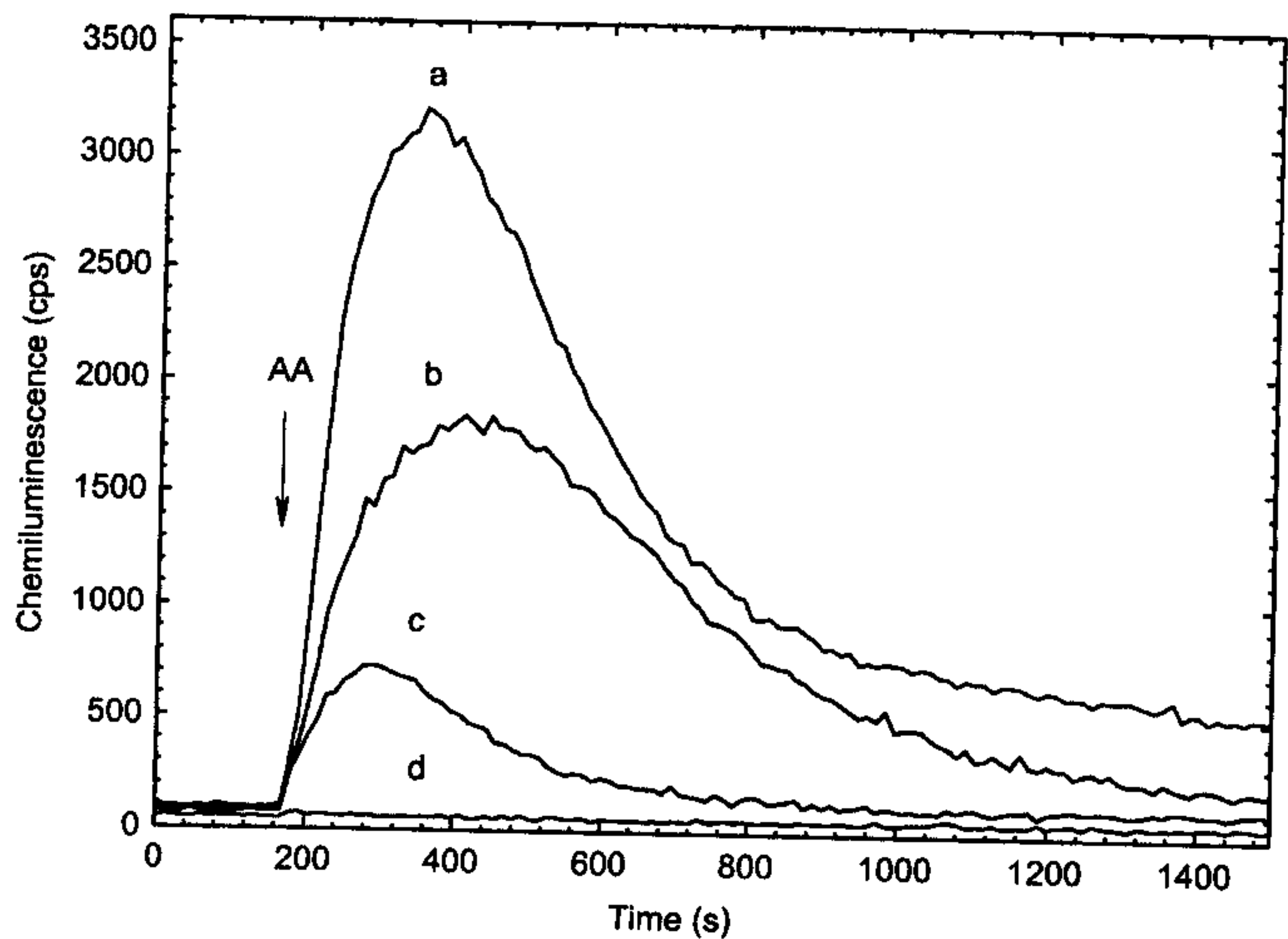


**Fig. 6.** Relative PI-3K activities in quiescent neutrophils and the cells stimulated by 5  $\mu\text{M}$  AA in the presence of various concentrations of wortmannin. The columns and the bands of  $^{32}\text{P}$ -labeled  $\text{PIP}_2$  correspond to quiescent cells (a) and stimulated cells treated with no wortmannin (b), 10 nM (c), 40 nM (d), and 100 nM wortmannin (e), respectively. Each data is the mean of three independent measurements, and the SD is indicated as the bar.

respiratory burst the same as that in other agonists, such as formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated respiratory burst. For this purpose, the AA-stimulated respiratory burst was measured in the neutrophils suspended in three different buffer solution:  $\text{Ca}^{2+}$ -containing HBSS;  $\text{Ca}^{2+}$ -free HBSS plus 2 mM EGTA, an effective chelator for extracellular calcium; and  $\text{Ca}^{2+}$ -free HBSS plus 2 mM EGTA and 5  $\mu\text{M}$  BAPTA/AM, an effective chelator for intracellular calcium. As shown in **Figure 7**, if the extracellular  $\text{Ca}^{2+}$  was chelated (cells were suspended in the  $\text{Ca}^{2+}$ -free buffer plus EGTA), the AA-stimulated respiratory burst was inhibited by ~70%. How-



**Fig. 7.** The role of intracellular and extracellular calcium in AA-stimulated respiratory burst in human neutrophils. The cells ( $10^6$  cells/ml) were suspended in HBSS containing 1.3 mM  $\text{Ca}^{2+}$  (a),  $\text{Ca}^{2+}$ -free HBSS containing 2 mM EGTA (b), and  $\text{Ca}^{2+}$ -free HBSS containing 2 mM EGTA plus 5  $\mu\text{M}$  BAPTA/AM (c) for 5 min, respectively, and were then stimulated with 5  $\mu\text{M}$  AA. The respiratory burst was measured as the concomitant chemiluminescence.



**Fig. 8.** The inhibitory effect of U73122 on AA-stimulated respiratory burst in human neutrophils. The cells ( $10^6$  cells/ml; HBSS containing 1.3 mM  $\text{Ca}^{2+}$  and 0.1  $\mu\text{M}$  luminol, pH 7.2) were preincubated with U73122 at 0  $\mu\text{M}$  (a), 0.1  $\mu\text{M}$  (b), 0.2  $\mu\text{M}$  (c), and 1  $\mu\text{M}$  U73122 (d), respectively, for 10 min and were then stimulated with 5  $\mu\text{M}$  AA at 37°C. The respiratory burst was measured as the concomitant chemiluminescence.

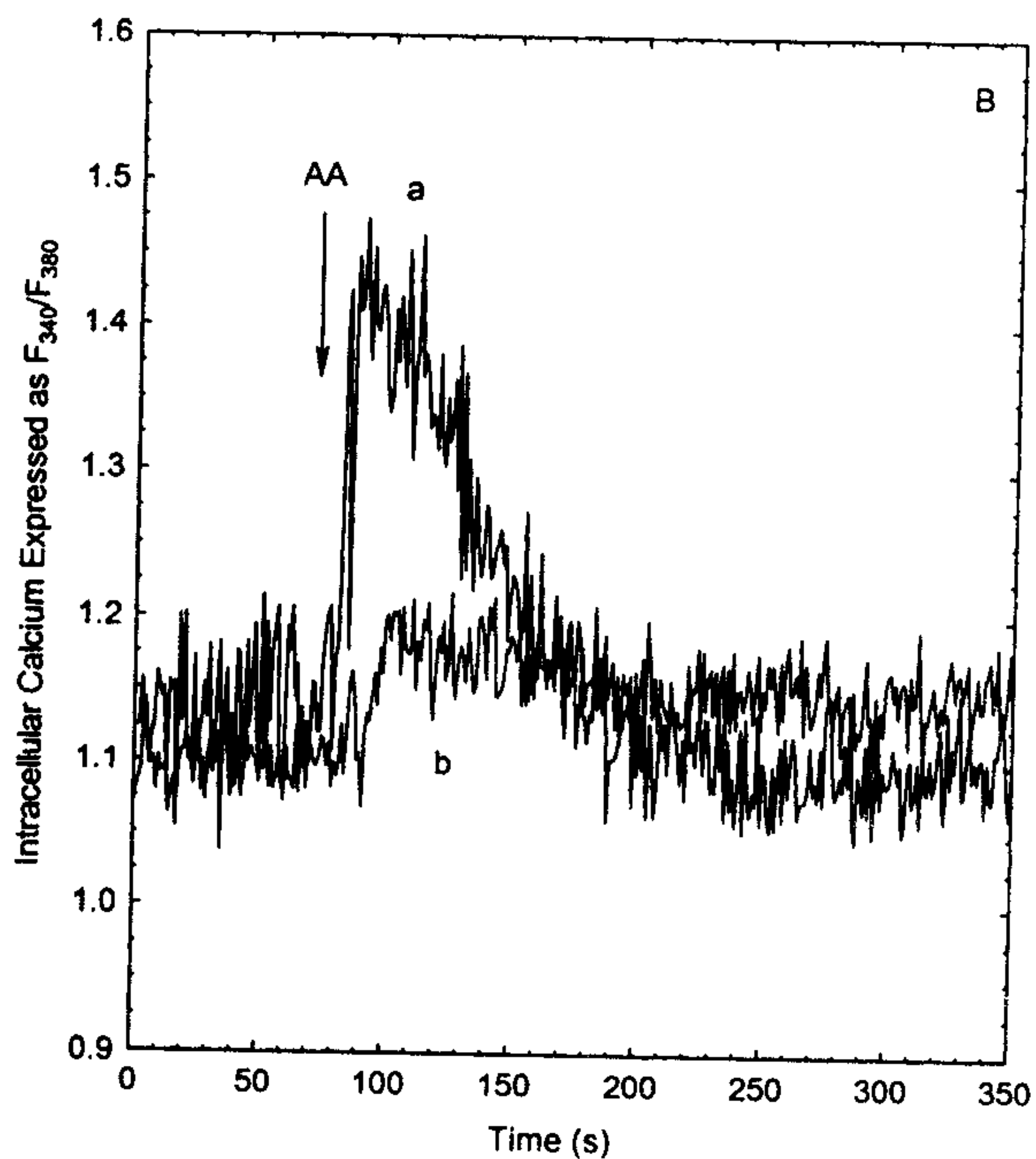
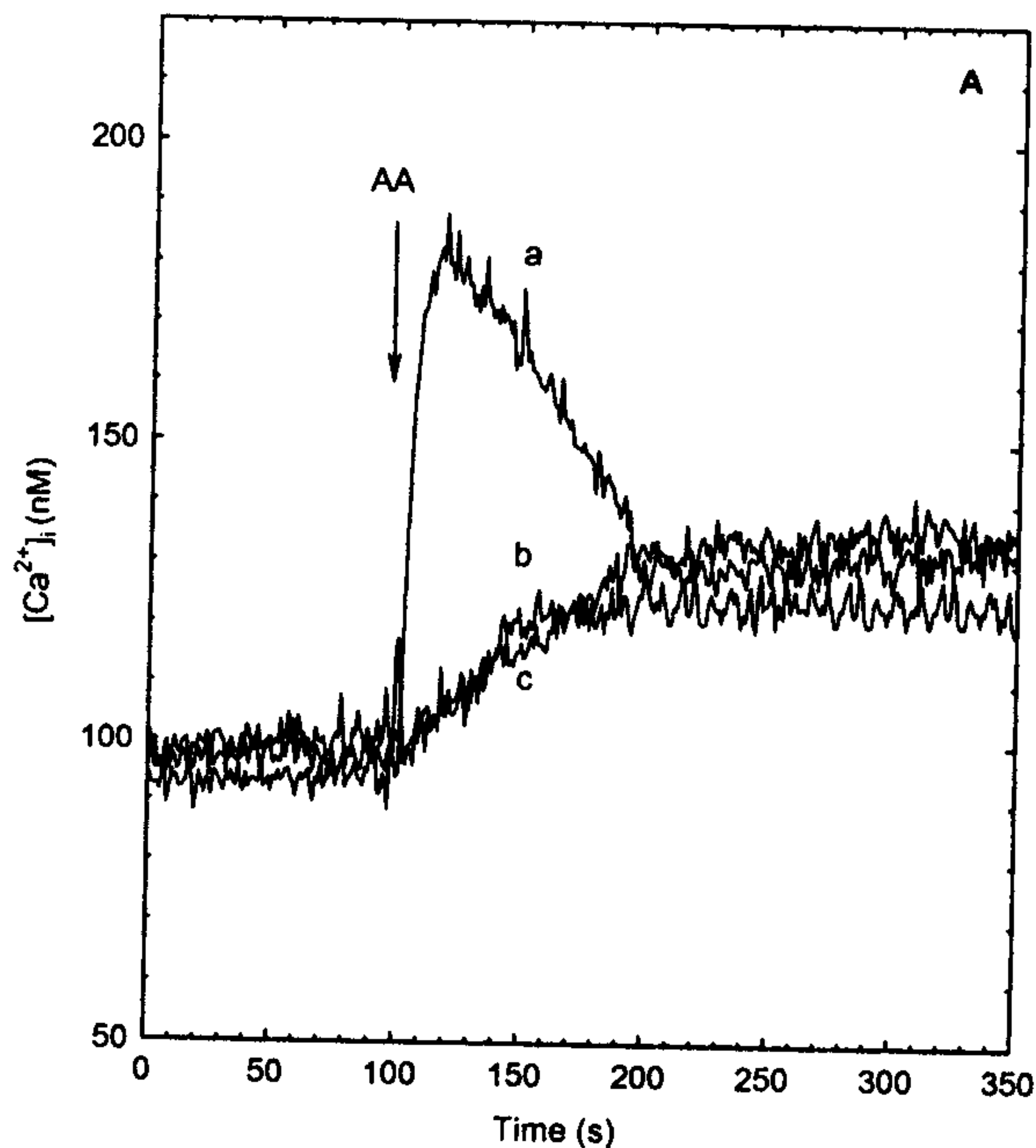
ever, if the intracellular- and extracellular-free  $\text{Ca}^{2+}$  were chelated (cells were suspended in the  $\text{Ca}^{2+}$ -free buffer plus EGTA and 5  $\mu\text{M}$  BAPTA/AM), the respiratory burst was inhibited by more than 90%. The results indicated that intracellular- and extracellular-free  $\text{Ca}^{2+}$  are important for AA-stimulated respiratory burst.

### The effect of U73122 on the AA-induced respiratory burst and calcium mobilization

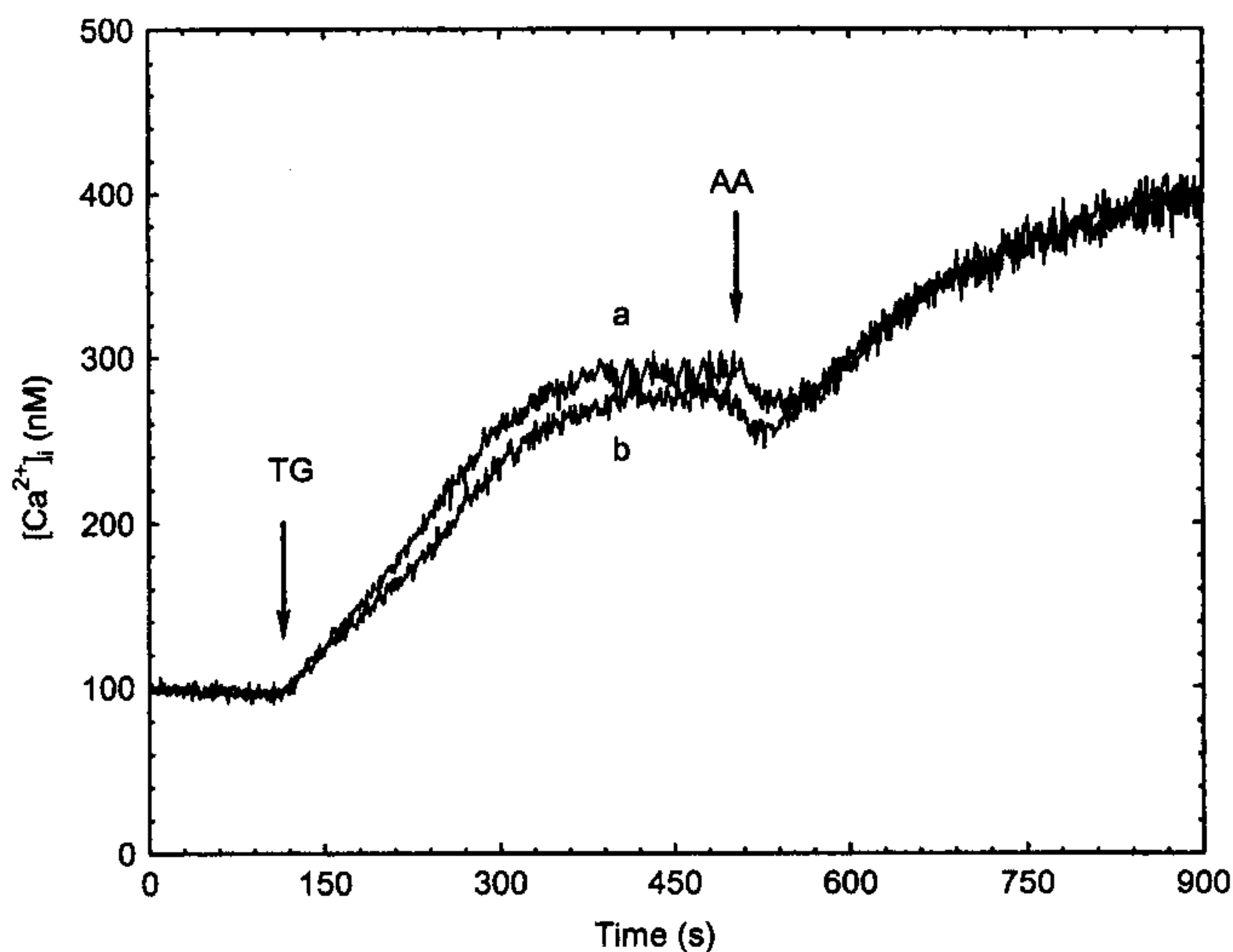
To demonstrate that PLC is actually involved in the AA-induced respiratory burst and  $\text{Ca}^{2+}$  mobilization in neutro-

phils, U73122, the inhibitor of PLC, was used. Cells were preincubated with U73122 of various concentrations for 10 min at 37°C and were then stimulated with 5  $\mu\text{M}$  AA. The recorded respiratory bursts are shown in **Figure 8**. It was found that the respiratory burst was inhibited by ~40% and 80% in the cells treated with 0.1  $\mu\text{M}$  and 0.2  $\mu\text{M}$  U73122, respectively. U73122 (1  $\mu\text{M}$ ) completely inhibited the burst. To verify the specificity of U73122 in inhibiting PLC activity, the effect of U73343, an inactive analog of U73122, on the AA-stimulated respiratory burst was also investigated. It turned out that even 5  $\mu\text{M}$  U73343 had no inhibitory effect on the respiratory burst. The concentration-dependent inhibitory effect of U73122 on the AA-stimulated respiratory burst further proves the involvement of PLC activation in the AA-induced respiratory burst of neutrophils.

The effect of U73122 on AA-induced cytoplasmic calcium mobilization was also investigated in the presence and absence of extracellular  $\text{Ca}^{2+}$ . As shown in **Figure 9A**, in contrast to the inhibitory effect of U73122 on AA-stimulated respiratory burst, AA can still induce calcium mobilization in the cells preincubated even with 2  $\mu\text{M}$  U73122. However, the kinetics of the mobilization in the cells preincubated with U73122 differs quite markedly from that observed in controlled cells. In U73122-treated cells, the AA-induced calcium mobilization becomes much more slow in rising and falling phases. It seems that when PLC activity was largely (or completely) inhibited by U73122, AA can still mobilize the calcium. It may mainly (or solely) come from the noncapacitative  $\text{Ca}^{2+}$  entry, as the  $\text{IP}_3$ -sensitive  $\text{Ca}^{2+}$  release from the internal store and the consequent capacitative  $\text{Ca}^{2+}$  entry were blocked. However, there may be some other possible source for the mobilization, as reported in literature [34]. Beaumier et al. [34] reported that in permeabilized human neutrophil, AA could mobilize a  $\text{Ca}^{2+}$



**Fig. 9.** The effect of U73122 on AA-induced cytoplasmic calcium mobilization in human neutrophils bathed in  $\text{Ca}^{2+}$  buffer or  $\text{Ca}^{2+}$ -free buffer. (A) The Fura-2-loaded cells ( $10^6$  cells/ml in HBSS containing 1.3 mM  $\text{Ca}^{2+}$ , pH 7.2) without treatment (curve a) or preincubated with 1  $\mu\text{M}$  U73122 (curve b) or 2  $\mu\text{M}$  U73122 (curve c) for 10 min were stimulated by 5  $\mu\text{M}$  AA at 37°C. (B) Similar measurements, except the  $\text{Ca}^{2+}$  buffer was replaced by  $\text{Ca}^{2+}$ -free buffer. The cells without treatment (curve a) and preincubated with 2  $\mu\text{M}$  U73122 (curve b) were stimulated.



**Fig. 10.** AA-induced  $\text{Ca}^{2+}$  entry in the neutrophils preincubated with TG. Six minutes after addition of 200 nM TG (curve a) or 100 nM TG (curve b), 5  $\mu\text{M}$  AA was added in the Fura-2-loaded cell suspension ( $10^6$  cells/ml, HBSS containing 1.3 mM  $\text{Ca}^{2+}$ , pH 7.2). Measurements were performed at 37°C.

pool that is insensitive to  $\text{IP}_3$ . To differentiate between these two possibilities, the effect of U73122 on the  $\text{Ca}^{2+}$  release in the cells bathed in  $\text{Ca}^{2+}$ -free buffer was also investigated. The results are shown in Figure 9B. It was found that U73122 (2  $\mu\text{M}$ ) could substantially but not completely inhibit the  $\text{Ca}^{2+}$  release in the absence of extracellular calcium. The small, remaining  $\text{Ca}^{2+}$  release indicates the existence of an  $\text{IP}_3$ -insensitive  $\text{Ca}^{2+}$  store. The fact that 1  $\mu\text{M}$  U73122 can completely inhibited the AA-induced respiratory burst but not the calcium mobilization in neutrophils suggests that without activation of PLC, AA is still able to induce cytoplasmic calcium mobilization through a noncapacitative  $\text{Ca}^{2+}$  entry or depletion of an  $\text{IP}_3$ -insensitive  $\text{Ca}^{2+}$  store.

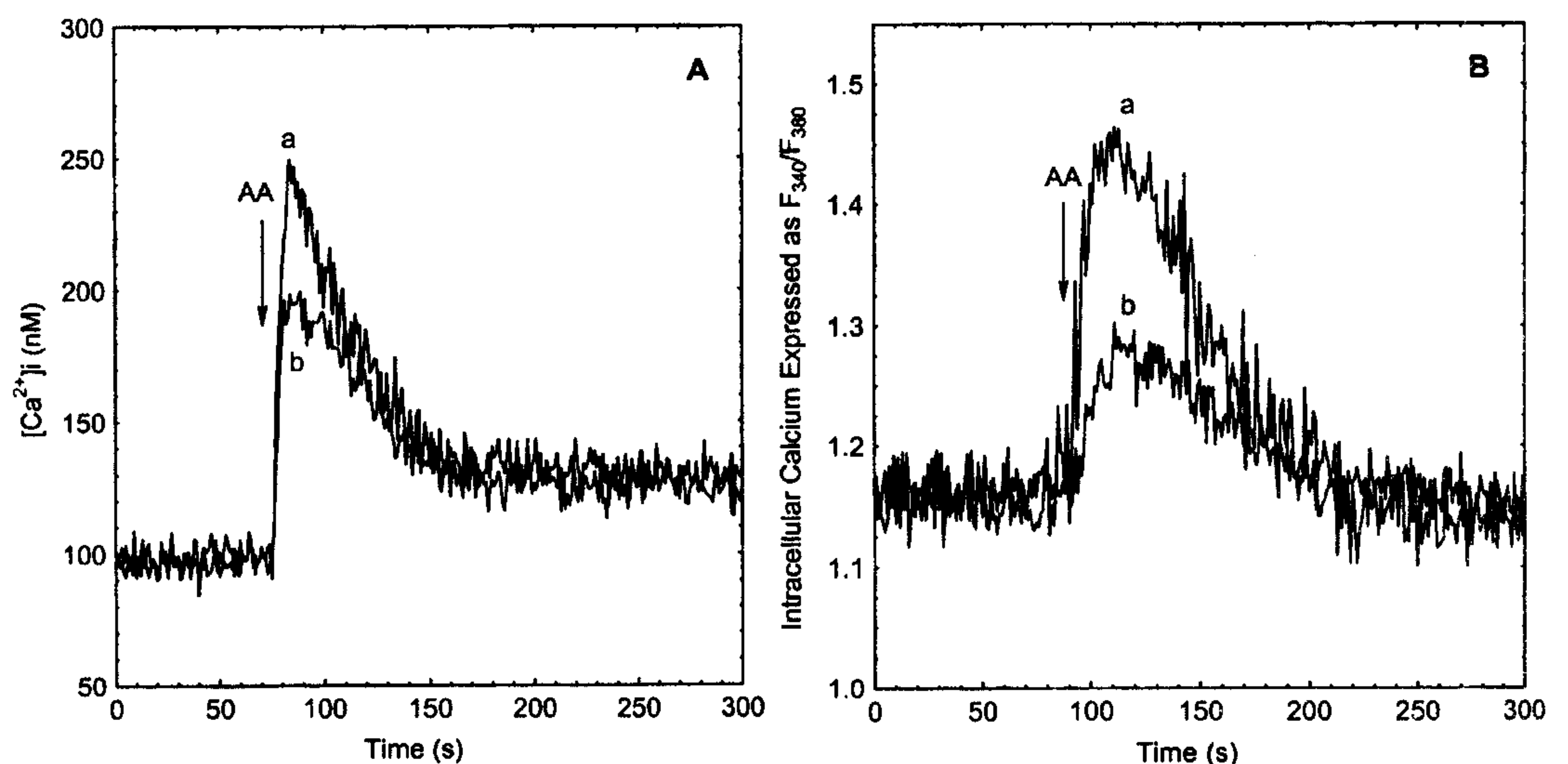
#### AA-induced $\text{Ca}^{2+}$ entry in the cells preincubated with thapsigargin (TG)

To further testify the point that AA can induce a noncapacitative  $\text{Ca}^{2+}$  entry in neutrophils, TG was added first in the cell

suspension containing  $\text{Ca}^{2+}$  to deplete the intracellular  $\text{Ca}^{2+}$  store and induce a capacitative  $\text{Ca}^{2+}$  entry, and then AA was added to see if an additional  $\text{Ca}^{2+}$  entry could be induced. The results are shown in **Figure 10**. It was observed that when intracellular  $\text{Ca}^{2+}$  concentration was elevated to a new plateau by adding 100 nM or 200 nM TG, the addition of 5  $\mu\text{M}$  AA did induce a new  $\text{Ca}^{2+}$  influx. It was also interesting to notice that the newly induced  $\text{Ca}^{2+}$  entry did not depend on the dose of the previously added TG, although 200 nM TG-induced capacitative  $\text{Ca}^{2+}$  entry was slightly higher than that induced by 100 nM TG. The fact that 200 nM TG was sufficient to deplete the intracellular  $\text{Ca}^{2+}$  store and that AA can still induce a new  $\text{Ca}^{2+}$  entry in the cells preincubated with the TG of such a high concentration provides strong evidence for the existence of the noncapacitative  $\text{Ca}^{2+}$  entry induced by AA.

#### The effect of wortmannin on $\text{Ca}^{2+}$ mobilization and the effect of U73122 on PI-3K activity in the AA-stimulated neutrophils

To know if there is any interaction or cross-talk between PI-3K signaling and PLC signaling, the effect of wortmannin, the inhibitor of PI-3K, on the cytoplasmic calcium mobilization and the effect of U73122, the inhibitor of PLC, on the PI-3K activity in the AA-stimulated cells were investigated. As shown in **Figure 11A**, when the neutrophils suspended in the HBSS  $\text{Ca}^{2+}$  buffer containing 100 nM wortmannin were stimulated by 5  $\mu\text{M}$  AA, the induced cytoplasmic  $\text{Ca}^{2+}$  mobilization was reduced by  $\sim 30\%$ . Based on three independent measurements, a  $27 \pm 9\%$  reduction was caused by 100 nM wortmannin. To further identify if the reduction of the cytoplasmic  $\text{Ca}^{2+}$  mobilization by wortmannin is attributed to the inhibition of  $\text{Ca}^{2+}$  release from an internal store, the same measurement was conducted in the  $\text{Ca}^{2+}$ -free buffer plus EGTA. As shown in **Figure 11B**, the  $\text{Ca}^{2+}$  release from the internal store is also partially inhibited by the presence of 100 nM wortmannin. The average reduction obtained in three independent measurements was  $59 \pm 6\%$ . These results indicate that the PLC activation or the production of  $\text{IP}_3$ , which induces the  $\text{Ca}^{2+}$  release from the intracellular store and subsequently, the store depletion-operated capacitative  $\text{Ca}^{2+}$  entry, may be regulated



**Fig. 11.** The effect of wortmannin on AA-induced cytoplasmic  $\text{Ca}^{2+}$  release and mobilization in human neutrophils. (A) The Fura-2-loaded cells ( $10^6$  cells/ml suspended in HBSS containing 1.3 mM  $\text{Ca}^{2+}$ , pH 7.4) were stimulated by 5  $\mu\text{M}$  AA without treatment (curve a) or after preincubation with 100 nM wortmannin for 10 min (curve b) at 37°C. (B) Similar measurements except the cells were suspended in  $\text{Ca}^{2+}$ -free HBSS buffer.

by PI-3K activity in AA-stimulated cells. To know if PLC activity exerts any influence on the PI-3K signaling, the PI-3K activities were measured in the neutrophils preincubated with 0.1 and 1  $\mu\text{M}$  U73122, respectively, and stimulated by 5  $\mu\text{M}$  AA. It turned out that no inhibitory effect of U73122 on the PI-3K activity was observed (data are not shown here).

## DISCUSSION

It has been well established that the activation of PLC leads to a generation of two second messengers,  $\text{IP}_3$  and DAG.  $\text{IP}_3$  induces a  $\text{Ca}^{2+}$  release from the intracellular calcium stores and consequently a capacitative  $\text{Ca}^{2+}$  entry operated by depletion of the stores [35]; DAG activates PKC. In the present study, the  $\text{IP}_3$  production in neutrophils was directly measured. It was found that the production of  $\text{IP}_3$  in AA-stimulated cells increased significantly in a dose-dependent manner (see Fig. 3). Meanwhile, the cytoplasmic  $\text{Ca}^{2+}$  mobilization was also observed in AA-stimulated neutrophils and showed a dose-dependent nature (see Fig. 2). The increased  $\text{IP}_3$  production and the dose-dependent cytoplasmic  $\text{Ca}^{2+}$  mobilization in AA-stimulated cells clearly indicate the activation of PLC in AA-stimulated neutrophils. The observed dose-dependent inhibition of the respiratory burst by U73122 (see Fig. 8) provides indirect evidence for the involvement of PLC activation in AA-induced respiratory burst of neutrophil. In this study, as observed in receptor-agonist fMLP-stimulated neutrophils [36], intracellular and extracellular  $\text{Ca}^{2+}$  are necessary for the respiratory burst in AA-stimulated cells. However, only 90% but not complete inhibition of the respiratory burst observed in the absence of intracellular and extracellular  $\text{Ca}^{2+}$  (see Fig. 7) suggests that some other signal pathway, which is independent on  $\text{Ca}^{2+}$  mobilization, may also be involved in AA-stimulated respiratory burst.

It has been recently reported that the AA can induce a noncapacitative  $\text{Ca}^{2+}$  entry in HEK293 cells [21–23]. In the present investigation, when U73122, at the concentration that was sufficient to completely suppress the AA-stimulated respiratory burst (1 and 2  $\mu\text{M}$  in the present study), was used to inhibit the  $\text{Ca}^{2+}$  released from the  $\text{IP}_3$ -sensitive internal stores and the subsequent capacitative  $\text{Ca}^{2+}$  entry, AA could still induce a  $\text{Ca}^{2+}$  entry (see Fig. 9A). The significant alteration in the kinetic pattern of AA-induced  $\text{Ca}^{2+}$  mobilization by U73122 clearly demonstrates the importance of the  $\text{Ca}^{2+}$  release from the  $\text{IP}_3$ -sensitive intracellular store for the AA-stimulated respiratory burst. The remaining  $\text{Ca}^{2+}$  entry may represent a noncapacitative entry or the  $\text{Ca}^{2+}$  mobilization from an unidentified  $\text{IP}_3$ -insensitive  $\text{Ca}^{2+}$  pool (see Fig. 9B). However, it seems unlikely that the relatively small  $\text{Ca}^{2+}$  release from the  $\text{IP}_3$ -insensitive pool could account for the relative large  $\text{Ca}^{2+}$  entry in the U73122-treated cells. To further verify the noncapacitative nature of the AA-induced  $\text{Ca}^{2+}$  entry, TG, a potent endomembrane  $\text{Ca}^{2+}$ -ATPase inhibitor, which can release  $\text{Ca}^{2+}$  from the intracellular store and induce a maintained elevation of intracellular  $\text{Ca}^{2+}$  by store depletion-operated  $\text{Ca}^{2+}$  influx [37], was used to deplete the intracellular  $\text{Ca}^{2+}$  store and induce the capacitative  $\text{Ca}^{2+}$  entry in cells suspended in  $\text{Ca}^{2+}$  buffer, and then AA was

added in the cell suspension to see if any additional  $\text{Ca}^{2+}$  entry could be induced. Fortunately, AA did induce a new  $\text{Ca}^{2+}$  influx after the intracellular  $\text{Ca}^{2+}$  concentration was elevated to a new plateau by the TG-induced capacitative  $\text{Ca}^{2+}$  entry. Experiments with U73122 and TG demonstrate the noncapacitative  $\text{Ca}^{2+}$  entry induced by AA in human neutrophils, although the role played by the  $\text{IP}_3$ -insensitive  $\text{Ca}^{2+}$  store cannot be definitely excluded. However, the fact that the AA induced noncapacitative  $\text{Ca}^{2+}$  entry but not respiratory burst in U73122-treated cells suggests that the AA-mediated, noncapacitative  $\text{Ca}^{2+}$  entry might not be an essential step for AA-stimulated respiratory burst in neutrophil.

In the present study, the PI-3K activity was assayed *in vitro* using PI(4)P as substrate, although the preferred *in vivo* substrate for class I PI-3Ks seems to be PI(4,5) $\text{P}_2$  [38]. As the class I PI-3Ks are able to phosphorylate *in vitro* PI, PI(4)P, and PI(4,5) $\text{P}_2$ , and class II PI-3Ks are restricted to PI and PI(4)P [39], the measured PI-3K activity in this study may be from class I and II subfamilies. The present investigation confirmed the report that activation of the PI-3K signaling pathway is involved in AA-stimulated human myeloid and endothelial cells [15]. The dose-dependence of respiratory burst and PI-3K activity on AA concentration and the inhibitory effect of wortmannin on AA-stimulated respiratory burst and PI-3K activity observed in this investigation (see Figs. 1 and 4–6) fairly well demonstrate the involvement of PI-3K signaling in the AA-stimulated respiratory burst in human neutrophil.

As PLC and PI-3K signaling pathways are involved in the AA-stimulated respiratory burst, and they use the PI(4,5) $\text{P}_2$  as a common substrate, it might be interesting to know if there is any interaction between these two pathways. To answer the question, “Does PLC activation influence PI-3K signaling?” the effect of U73122 on PI-3K activity was determined. It turned out that inhibition of PLC activity did not affect the activation of PI-3K in the AA-stimulated neutrophils. To answer another question, “Does PI-3K activity regulate PLC signaling?” the effect of wortmannin, the selective inhibitor of PI-3K, on PLC activation-derived cytoplasmic  $\text{Ca}^{2+}$  mobilization was investigated. It was found that inhibition of PI-3K activity by wortmannin resulted in a partial inhibition of the  $\text{Ca}^{2+}$  mobilization. The reduced cytoplasmic  $\text{Ca}^{2+}$  mobilization, in particular, the reduced  $\text{Ca}^{2+}$  release from internal stores, in the wortmannin-treated cells (shown in Figs. 11A and B), suggests that a certain portion of PLC activity is gained via activation of PI-3K. However, the fact that wortmannin at the concentration, which is sufficient to completely inhibit PI-3K activity in the AA-stimulated cells, only reduces  $\sim 50\%$  of the  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  release from the intracellular store implies that the activation of a considerable portion of PLC is independent on the PI-3K activation in the AA-stimulated neutrophils. As reported by Cantley and Schlessinger and co-workers [26, 27], the lipid products of PI-3K, PI 3,4,5,-trisphosphate [PI(3,4,5) $\text{P}_3$ ], can bind to the C-terminal Src homology 2 domain or pleckstrin homology domain of PLC $_{\gamma}$ , causing translocation of PLC $_{\gamma}$  from cytosol to plasma membrane. Such a membrane-targeting effect of PI(3,4,5) $\text{P}_3$  makes PLC $_{\gamma}$  available for activation. This may be the mechanism involved in the PI-3K-dependent PLC activation, which may account for the



inhibition of the  $\text{Ca}^{2+}$  mobilization by wortmannin in AA-stimulated cells.

To date, it has been known that the PLC family can be classified into three major subfamilies:  $\beta$ -,  $\gamma$ -, and  $\delta$ -isozymes [40]. Stimulation of PLC $\beta$  isozymes by many agonists occurs through the receptors coupled to heterotrimeric G-proteins [41]. The involvement of PLC $\beta$  in AA-stimulated respiratory burst has been implicated in some previous reports. For example, pertussis toxin, the inhibitor of GTP-binding protein, inhibited AA-induced  $\text{O}_2^-$  generation in intact neutrophils [13] and the transient increase in cytosolic-free  $\text{Ca}^{2+}$  in human airway smooth muscle cells as well [20, 42]. PLC $\gamma$  may be activated by tyrosine phosphorylation or by various lipid-derived second messengers [43]. In the former case, a number of stimuli activate PLC $\gamma$  through the activation of protein tyrosine kinases [40, 44]. In the latter case, the activation of PLC $\gamma$  is independent on tyrosine phosphorylation but via binding to PI(3,4,5)P $_3$  [27] or by some lipid metabolites, such as phosphatidic acid and AA, generated by the action of other extracellular signal-regulated effector enzymes [45, 46]. Although the precise mechanisms, through which the respiratory burst in neutrophils is stimulated by AA cannot be very well defined at the present time, the following conclusions may be drawn: PLC and PI-3K signaling are activated by AA in human neutrophils. The AA may initiate the PLC signaling pathway by activating PLC $\beta$  and PLC $\gamma$ . The PLC $\beta$ -activated pathway is likely independent on PI-3K activation, and the cytoplasmic  $\text{Ca}^{2+}$  mobilization induced by PLC $\beta$  activation may not be inhibited by the inhibitor of PI-3K. However, the PLC $\gamma$ -activated pathway is dependent on PI-3K activation. The observed inhibition of the cytoplasmic  $\text{Ca}^{2+}$  mobilization, in particular, the  $\text{Ca}^{2+}$  release from the internal store, by the PI-3K inhibitor may represent the calcium mobilization caused by PLC $\gamma$  activation in AA-stimulated cells.

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