THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 52, Issue of December 28, pp. 49093–49099, 2001
Printed in U.S.A.


# Inhibition of Phosphatidylinositol 4-Kinase Results in a Significant Reduced Respiratory Burst in Formyl-methionyl-leucyl-phenylalanine-stimulated Human Neutrophils*

Received for publication, February 12, 2001, and in revised form, October 7, 2001
Published, JBC Papers in Press, October 9, 2001, DOI 10.1074/jbc.M101328200

**Jiachang Yue‡§, Jiang Liu§¶, and Xun Shen¶‖**

*From the ¶Department of Cell Biophysics and the ‡National laboratory of Biomolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China*

**The effects of phenylarsine oxide and a monoclonal antibody directed against type II phosphatidylinositol 4-kinase (PI4K) on the *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated respiratory burst and the PI4K activity in neutrophils were investigated. Fluorescence microscopic imaging showed that the antibody labeled with IANBD amide (*N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine) could enter into the cytosol possibly by endocytosis. It was found that the antibody inhibited the fMLP-stimulated respiratory burst but had little effect on the phorbol myristate acetate-activated respiratory burst in neutrophils, whereas phenylarsine oxide inhibited both. It was found that even at higher concentration, the antibody could not completely inhibit the cell response. Using cells preincubated with human immunoglobulin G of the same concentration as the control, the maximal inhibition of the fMLP-stimulated respiratory burst by the antibody against type II PI4K was found to be about 70%, whereas the PI4K activity was inhibited by only about 40%. The discrepancy in depressing the cell response and the enzyme activity may be the result of depletion of the phosphatidylinositol 4,5-bisphosphate or phosphatidylinositol 3,4,5-trisphosphate pools during the incubation of cells with the antibody. Both the 40% inhibition of PI4K activity and 70% depression of the respiratory burst by the type II PI4K antibody may imply that at least 40% of the phosphatidylinositol 4,5-biphosphate was synthesized promptly by all forms of PI4K and phosphatidylinositol-4-phosphate 5-kinase in the fMLP-activated cells. The results suggest that PI4K plays a central role in either phospholipase C or PI3K signaling and that PI3K, PI4K, and phosphatidylinositol 4-phosphate 5-kinase must be considered as an integrated family for the phosphatidylinositol 3,4,5-trisphosphate initiated signaling.**

Chemoattractant-mediated recruitment of leukocytes is a key step in the process of inflammation. Chemokines and chemotactic peptides, such as *N*-formyl-methionyl-leucyl-phenylalanine (fMLP),[1] complement fragment C5a, and interleukin-8, bind to G protein-coupled receptors, then trigger a series of signaling events in cell movement, phagocytosis, degranulation, and superoxide generation (1, 2). It is known that two signaling pathways mediated by phospholipase C (PLC) (3) and phosphatidylinositol 3-kinase (PI3K) (4, 5) are activated by chemoattractant receptors. The PLC pathway involves two important second messengers, inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol, released by cleavage of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$). The PI3K pathway involves the activation of some membrane-trafficking proteins and enzymes such as the phosphoinositide-dependent kinases, protein kinase B, and S6 kinases by the key second messenger phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$) (6, 7). $PI(3,4,5)P_3$ is generated from $PI(4,5)P_2$ through phosphorylation at the D3 position of the inositol ring by PI3Ks that are linked to tyrosine-based receptors or G proteins. A fundamental link between the two distinct signaling pathways results because each utilizes primarily the same substrate, $PI(4,5)P_2$. However, $PI(4,5)P_2$ is the product of phosphatidylinositol 4-kinases (PI4Ks) and phosphatidylinositol 4-phosphate 5-kinases (PIP5Ks). PI4K catalyzes the phosphorylation of phosphatidylinositol at the 4′ position to produce phosphatidylinositol 4-monophosphate (PI(4)P), which is then converted to $PI(4,5)P_2$ by PIP5K (8). Because synthesis, degradation, and subsequent resynthesis of the phosphoinositides form a metabolic cycle, it should be expected that there might be equilibrium among $PI(4,5)P_2$, PI(4)P, and PI in resting cells. If PLC and PI3K were activated by the fMLP stimulation in cells such as neutrophils, was there enough $PI(4,5)P_2$ available for initiating the PLC pathway or PI3K pathway fully? In other words, is a concomitant activation of PI4K needed to produce additional PI(4)P and its converted $PI(4,5)P_2$ for the activated PLC or PI3K to utilize? Up until now, there has been no such an investigation reported.

There have been a number of investigations showing the key role played by PI3K in the fMLP-activated neutrophil (9–12). However, little is known about the role of PI4K in the activa-

* This work was supported by National Natural Science Foundation of China Grants 39970197 and 19890380-4. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ These authors contributed equally to this work.

‖ To whom correspondence should be addressed: Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Rd., Chaoyang District, Beijing 100101, People's Republic of China. E-mail: shenxun@sun5.ibp.ac.cn.

[1] The abbreviations used are: fMLP, formyl-methionyl-leucyl-phenylalanine; PLC, phospholipase C; PI, phosphatidyl inositol; PI3K, phosphatidylinositol 3-kinase; PI4K, phosphatidylinositol 4-kinase; $IP_3$, inositol 1,4,5-trisphosphate; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; $PI(3,4,5)P_3$, phosphatidylinositol 3,4,5-trisphosphate; PI4K, phosphatidylinositol 4-kinase; PIP5K, phosphatidylinositol 4-phosphate 5-kinase; PI(4)P, phosphatidylinositol 4-monophosphate; PAO, phenylarsine oxide; PMA, phorbol myristate acetate; IANBD amide, *N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine; HBSS, Hanks' balanced salt solution; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; $PIP_2$, phosphatidylinositol bisphosphate; $PIP_3$, phosphatidylinositol trisphosphate.

tion of neutrophils. To study PI4K, some specific inhibitor of PI4K may be necessary. Wortmannin is known to be an effective inhibitor of PI3K and has been employed as an alternative mode to classify PI4K. It was also reported that multiple isoforms of PI4K existed (13). Some of the isomers, such as type III enzymes, PI4K230 and PI4K92, are wortmannin-sensitive and can be inhibited half-maximally at a higher concentration of wortmannin (submicromolar concentration) (14, 15). Some of the isomers, such as type II enzyme, the 55-kDa forms, are insensitive to wortmannin (16). The wortmannin-sensitive and -insensitive PI4K might both be involved in the agonist-stimulated polyphosphoinositide turnover. Although wortmannin could inhibit the activity of some isoforms of PI4K, its strong inhibitory effect on PI3K would obscure the role of PI4K. Phenylarsine oxide (PAO) has been used as an inhibitor of PI4K to examine the participation of PI4K in nerve growth factor retrograde axonal transport within sympathetic and sensory neurons (17). It was reported that PAO inhibited the production of PI(4)P by interfering with the activity of PI4K (18) and inhibited the endocytosis of the muscarinic cholinergic receptor in SH-SY5Y neuroblastoma cells by inhibiting PI4K activity (19). However, the specificity of PAO was not checked carefully. In this study, PAO and a monoclonal antibody directed against type II PI4K were used to study the role of type II PI4K in the fMLP-simulated respiratory burst in human neutrophils. The specificity of both PAO and the monoclonal antibody was investigated.

## EXPERIMENTAL PROCEDURES

*Materials*—fMLP, dextran T-500 (molecular weight 500,000), phorbol myristate acetate (PMA), PI, PI(4)P, polyethylene glycol (molecular weight 1,500), phenylarsine oxide (PAO), and dithiothreitol were purchased from Sigma. IANBD amide was from Molecular Probes. Human immunoglobulin G (IgG) was obtained from the Military Academy of the Medical Sciences (Beijing, China). Lymphocyte separation solution (with a density of 1.007 ± 0.002) was from the Institute of Hematology, Chinese Academy of Medical Sciences (Tianjing). [$\gamma$-$^{32}$P]ATP was from the Yan-Huai Company (Beijing). All other reagents used were of analytical grade.

*Isolation of Neutrophils*—Human neutrophils were isolated from the blood of healthy donors according to the following procedure. Blood was mixed with 4.5% dextran in a 0.9% NaCl solution. The red cells were settled at room temperature for 40 min, and then leukocyte-rich plasma was layered on the top of the lymphocyte separation 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 Hanks' balanced salt solution (HBSS) containing 1 mM $CaCl_2$ (pH 7.4).

*Chemiluminescence Measurement of Respiratory Burst*—The respiratory burst of neutrophils was monitored by chemiluminescence methods in which the emitted light from the reaction of luminol with the superoxide anion ($O_2^-$) and $H_2O_2$ generated by cells was detected as a concomitant chemiluminescence burst. Each 2-ml neutrophil suspension ($10^6$ cells/ml) containing 0.1 $\mu$g of luminol was added to a quartz cuvette. Two identical cuvettes containing testing or control cell suspension were placed in a rotatable sample holder of a laboratory-made photon counter and measured at 37 °C. Because the respiratory bursts of the cells for test and control were measured simultaneously, the errors induced by the time-dependent variation in cell vitality, intensity, and kinetics of respiratory burst were minimized to a great extent.

*Preparation of Monoclonal Antibody*—High yield purified PI4K was obtained from bovine cerebella cortex using the method described by Scholz *et al*. (20). The purified PI4K was categorized as a type II 55-kDa enzyme by measurement of the apparent $K_m$ of the enzyme with PI and ATP, wortmannin test, and molecular weight analysis, which was reported elsewhere (21). The antibody named A6D was prepared according to the method described by Endemann (22) with some minor modification. 100 $\mu$l of complete Freund's adjuvant was vortex mixed with an equal volume of 1 mg/ml purified PI4K solution for 5 min. The mixture was injected into the backs of 4-week-old BALB/c female mice. The mice were booster immunized by injecting a mixture of incomplete Freund's adjuvant with the PI4K into their backs 3 weeks later. After another 2 weeks, 50 $\mu$g of PI4K in phosphate-buffered saline (PBS) was injected directly into the spleens of the mice. The spleens were removed 3 days later, then splenic lymphocytes were harvested and fused with SP2/0 mice myeloma cells at a ratio of 2:1 in PBS using 50% polyethylene glycol as a fusion agent. The fused cells were cultured in 96-well plates at an appropriate density, and the hybrids were selected by Dulbecco's modified Eagle's medium containing 2% hypoxanthine, aminoptrein, and thymidine. The supernatants of hybrid cells were screened for the presence of antigens by indirect enzyme-linked immunosorbent assay. The high positives were propagated three or four times and subcloned by limit dilution. Approximately $10^6$ cloned hybridoma cells were injected intrapertoneally into mineral oil-primed BALB/c mice, and ascites were tapped out 10–15 days later. Antibodies were precipitated from ascites by 40% ammonium sulfate, then dissolved, dialyzed, and purified further on DEAE-10 fast protein liquid chromatography columns. The active fraction was pooled and concentrated to 2 ml (1 mg/ml) in PBS. The antibody was named A6D, and its molecular mass was determined by SDS-PAGE. It contains a 55-kDa heavy chain and a 25-kDa light chain. The mass of the antibodies was ~160 kDa. The subisotype of the antibody was determined to be $IgG_{2b}$ using a kit from Hyclone. The purity of the antibody was better than 95%. The specificity of the antibody toward PI4K was checked carefully by Western blotting and is reported elsewhere (23).

*Assay of PI4K Activity in Neutrophils*—The PI4K activity was assayed by measuring the incorporation of the label $^{32}$P into PI(4)P (15, 25). Briefly, 100 nM fMLP was added to a 2-ml neutrophil suspension ($3 \times 10^6$ cells/ml) preincubated with PAO or the antibody or human IgG at 37 °C to stimulate the respiratory burst of the cells; 40 s later the suspension was cooled rapidly in an ice bath. After centrifugation, the cells were lysed with 500 $\mu$l of lysing buffer containing 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1% Triton X-100, 5 mM $CaCl_2$, 10 mM PIPES, 1.2 mM phenylmethylsulfonyl fluoride, 1 $\mu$g/ml leupeptin, 1 $\mu$g/ml aprotinin, 10 mM NaF, and 5 mM EDTA (pH 6.8) for 7 min at 4 °C. The nuclear matrices were eliminated from the cell lysate by centrifugation at 650 × *g* for 5 min. Then ammonium sulfate (final concentration of 0.25 M) was added to the supernatant, which contained membrane fraction, to allow the proteins contained in the supernatant to be precipitated for 40 min at 4 °C. The precipitate containing PI4K was then pelleted at 10,000 × *g* for 20 min. The pellet was dissolved with 45 $\mu$l of assay buffer (50 mM Tris-HCl, 2 mM EGTA, 2 mM EDTA, 10 mM $Mg^{2+}$, 0.4% Triton X-100, 2 $\mu$M phenylmethylsulfonyl fluoride, 1 $\mu$g/ml leupeptin, 1 $\mu$g/ml aprotinin, 200 $\mu$g/ml sonicated PI, 2 $\mu$Ci of [$\gamma$-$^{32}$P]ATP, and 25 $\mu$M normal ATP) at 37 °C for 10 min. The reaction was stopped by adding 100 $\mu$l of 1 N HCl, then the lipids in the mixture were extracted with 300 $\mu$l of $CHCl_3$:MeOH (2:1, v/v). The organic layer was collected, washed twice with a mixture of 1 N HCl and methanol (1:1, v/v), and finally analyzed by thin layer chromatography on silica gel plates (TLC aluminum sheets, Merck KgaA, Germany) using authentic PI(4)P as a reference. The gel plates were developed in a mixture of chloroform, methanol, 25% ammonium hydroxide, and water (70:100:15:25, v/v). The radioactivity of the $^{32}$P-incorporated PI(4)P bands on the TLC sheet was measured either by the Storm 820 Imaging System (Amersham Pharmacia Biotech) using storage Phosphor technology (in the case of the antibody and IgG) or by liquid scintillation counting (in the case of PAO). All assays were performed in duplicate.

*Labeling the Antibody with IANBD Amide*—Referring to the method described by Shore at al. (26), the antibody against PI4K was labeled with the fluorescence probe IANBD amide as follows. The purified A6D was dissolved in 100 $\mu$l of PBS containing 1 mM dithiothreitol (pH 7.4) at a concentration of 0.5 mg/ml. 150 mM IANBD amide was added to the solution to react with the antibody for 8 h at 4 °C in the dark. The reaction mixture was then dialyzed three times in PBS to remove free IANBD amide. The IANBD-labeled antibody was checked fluorometrically; its maximal excitation wavelength was at 478 nm and emission maximum at 540 nm.

*Microscopic Imaging of Fluorescence-labeled Antibody Entering the Cells*—1.0 ml of neutrophil suspension ($5 \times 10^5$ cells/ml in HBSS) was transferred to a glass-bottomed dish and incubated for 30 min at 37 °C. The fluorescence (excitation, 478 nm; emission, above 520 nm) of the attached single cells on bottom glass of the dish before and after the addition of IANBD-labeled antibody was monitored under a Nikon Diaphot 300 inverted fluorescence microscope equipped with an Aqua-Cosmos Microscopic Image Acquisition and Analysis System provided by Hamamatsu Photonics K.K. (Japan). The digitized fluorescence images of cells and the kinetic change of the fluorescence in single cells were obtained and processed on-line on a computer. After the fluorescence from the cells reached higher steady-state levels, $CuSO_4$, whose concentration was 10-fold that of the IANBD concentration, was added

to the dish to quench the fluorescence from the labeled antibody adhering to the cell surface.

## RESULTS

*Effect of PAO on the fMLP- and PMA-stimulated Respiratory Burst in Neutrophils*—Different concentrations of PAO (from 200 nM to 1 mM) were added to the cell suspension 5 min before the neutrophils were stimulated with 200 nM fMLP, and the corresponding respiratory bursts were recorded. It was found that 200 nM PAO inhibited the burst by about 60%, and 600 nM PAO reduced the burst by a factor of 3 compared with the control. 1 mM PAO was found to inhibit the respiratory burst almost completely.

To know whether the action of PAO takes place at a step upstream or downstream from calcium mobilization, the effect of PAO on the PMA, a direct activator of protein kinase C, stimulated respiratory burst was also investigated. The results show that PAO inhibits the respiratory burst stimulated by PMA too, but the concentration needed to achieve the same inhibition as that observed in fMLP stimulation is obviously higher. For instance, 1 mM PAO inhibited the PMA-stimulated respiratory burst only by about 70%, whereas it inhibited the fMLP-stimulated burst completely. The results (not shown here) indicate evidently that PAO inhibits not only the PI4K activity but also the activity of protein kinase C. Therefore, it is not a specific inhibitor for PI4K.

*Uptake of the Monoclonal Antibody against PI4K by Neutrophils*—Because the antibody A6D is a high molecular mass protein (160 kDa), whether it can enter into the cells and inactivate PI4K is extremely important for this study. Fluorescence microscopy was used to observe whether the fluorescent IANBD-labeled A6D could be accumulated within the cells after its addition into the neutrophil-attached dishes. As described under "Experimental Procedures," 8 $\mu$g/ml IANBD-labeled A6D was added to the cell-attached glass-bottomed dish while imaging the cells with the digital fluorescence microscope. A typical result from one of five observations is shown in Fig. 1. The pseudo-color images of the cells taken at various moments after the addition of the IANBD-labeled antibody into the observed dish showed that the fluorescent antibody gradually appeared inside the cells and localized mainly in cytosolic volume. The fluorescence from the cells increased with the time after the addition of the fluorescence-labeled A6D in the suspension. The bright fluorescence from the cells in contrast with the dark background observed by the microscope may suggest that the fluorescence observed should come from the antibody entered in the cells. To verify such a speculation, 0.2 mM $CuSO_4$, a concentration $4 \times 10^3$ times higher than that of the IANBD-labeled antibody, was added to the dishes to quench the fluorescence (27), which may come from the labeled antibody adhered on cell surface, after the apparent cell fluorescence reached a higher plateau. Fortunately, the added copper sulfate did not notably quench the fluorescence from the cells. The *lower panel* in Fig. 1 shows the kinetic change of the fluorescence from the labeled antibody in five randomly selected cells. It clearly demonstrates that the cells do take up the high molecular mass antibody, and $CuSO_4$, the effective quencher of IANBD fluorescence, at a relatively much high concentration did not quench the fluorescence even 5 min after its addition. The microscopic images clearly show that the antibody can really enter into the cells.

*Time-dependent Inhibition of the fMLP-stimulated Respiratory Burst by the Monoclonal Antibody against PI4K*—Because microscopic imaging shows that the uptake of the antibody by cells is time-dependent, it should be expected that the inhibition of the fMLP-stimulated respiratory burst by the antibody might be also time-dependent. The effect of the antibody on the

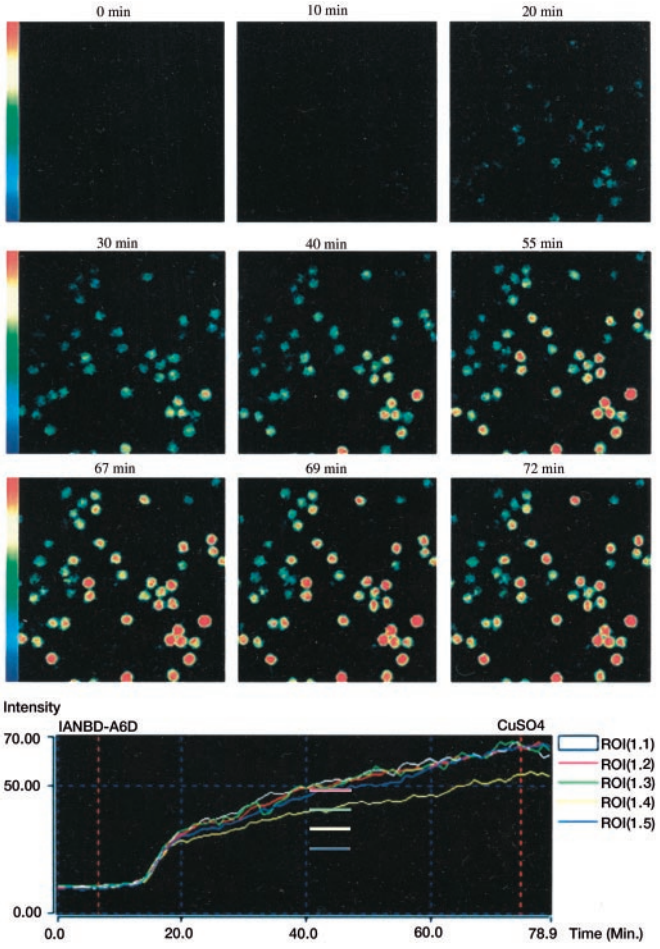


FIG. 1. **The microscopic fluorescence pseudo-colored images of the neutrophils at various indicated moments after incubation with the IANBD-labeled antibody.** 1.0 ml of neutrophil suspension ($5 \times 10^5$ cells/ml in HBSS) was transferred to a glass-bottomed dish and incubated for 30 min at 37 °C. Then, the microscopic fluorescence images were taken every min, and each image was acquired for 200 ms at an excitation wavelength of 478 nm and emission wavelength longer than 520 nm. 8 $\mu$g/ml IANBD-labeled antibody was added to the dish 7 min after the measurement started. 0.2 mM $CuSO_4$ was added to the dish 74 min after the measurement started. The *lowest panel* shows the kinetic changes of the fluorescence in five cells selected from the *lower right corner* of the microscopic field.

fMLP-stimulated respiratory burst was measured in the cells (1 [×] $10^6$ cells/ml) preincubated with 20 ng/ml antibody for various times from 0 to 90 min using the cells preincubated with human IgG of the same concentration for the same period of time as a control. It was found that no inhibition of the respiratory burst was found in the cells stimulated immediately after the addition of the antibody in cell suspension. However, as the preincubation increased, a more and more obvious inhibitory effect of the antibody on the cell response was observed. The results are shown in Fig. 2 as *curve a*. It shows that inhibition of the fMLP-stimulated respiratory burst rises with the preincubation time in an exponential-like manner; about 20% and 55% inhibition were found in the cells preincubated for 20 and 40 min, respectively. After 60 min the respiratory burst was inhibited by about 70% and was not reduced further. The results clearly indicate that a certain preincubation time was needed for the antibody to enter into the cells and exert its effect.

*Concentration Dependence of the Inhibitory Effect of the Monoclonal Antibody against PI4K on the fMLP-stimulated Respiratory Burst*—Inhibition of the fMLP-stimulated respiratory burst in the neutrophils by the PI4K antibody of different

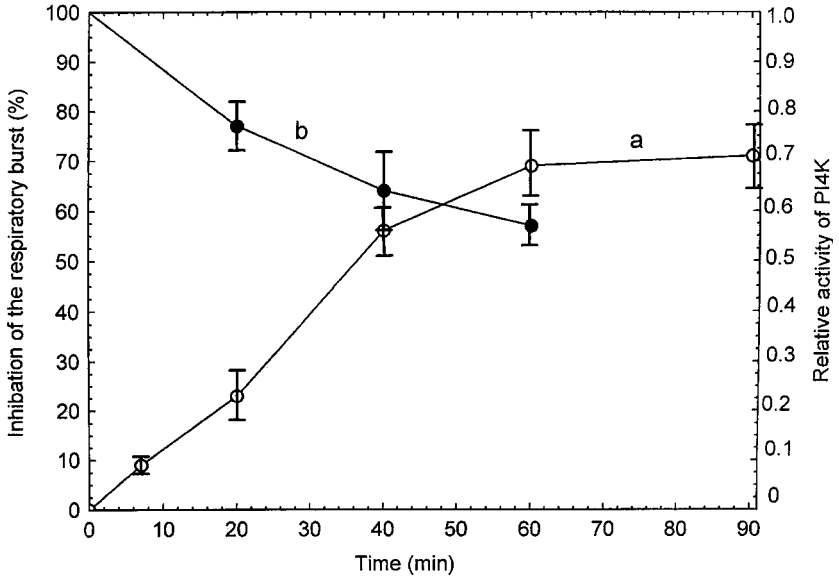


FIG. 2. **Inhibition of the fMLP-stimulated respiratory burst and the PI4K activity in the stimulated cells preincubated with the PI4K antibody for various times.** *Curve a*, $1 \times 10^6$ cells/ml were put into two cuvettes. The cells in one cuvette were incubated with 20 ng/ml antibody, and the cells in another cuvette were incubated with human IgG of the same concentration for 0, 7, 20, 40, 60, and 90 min before stimulation by 200 nM fMLP. Then the fMLP-stimulated respiratory bursts in the two cuvettes were measured simultaneously using a rotating sample holder. The inhibition was calculated using the respiratory bursts in the cells preincubated with IgG as controls. Respiratory bursts were measured as the concomitant chemiluminescence at 37 °C. *Curve b*, the PI4K activities measured in the fMLP-stimulated neutrophils preincubated with 20 ng/ml antibody for 20, 40, and 60 min. The data were obtained from two independent assays. The relative activity was calculated using the PI4K activity measured in untreated cells. All of the data are the mean of two independent measurements.

concentrations was investigated. The respiratory bursts in the cells ($1 \times 10^6$ cells/ml) preincubated with 1, 4, 20, and 100 ng/ml A6D for 70 min were measured using the bursts measured simultaneously in the cells preincubated with human IgG of the corresponding concentrations as for the controls. Four sets of the measurements are shown in Fig. 3. It was observed that the respiratory burst was depressed more significantly with an increase of the A6D concentration. However, when the antibody concentration exceeded 20 ng/ml, further inhibition was very limited. It differs from PAO; the antibody cannot completely inhibit the fMLP-stimulated respiratory burst even at a higher concentration. This may indicate that even though all type II PI4Ks were inactivated by binding to its antibody, a weak respiratory burst (about 30% of the response in the human IgG-treated cells) can be still stimulated by fMLP. To know whether the inhibition is really caused by inactivation of the PI4K by binding to its antibody rather than a nonspecific antibody effect, the fMLP-stimulated respiratory bursts in the cells preincubated with human IgG of the corresponding concentrations were measured simultaneously and used as controls. The stimulated respiratory bursts in the cells preincubated with human IgG of 20 and 100 ng/ml are shown in Fig. 4. It was found that IgG at the concentration below 4 ng/ml almost did not inhibit the cell response, and 20 and 100 ng/ml IgG inhibited the burst by about 10 and 25%, respectively.

*Effect of the PI4K Antibody on the PMA-stimulated Respiratory Burst*—To examine whether the inhibition of the respiratory burst by the antibody is the result of its specific action on PI4K or its interference with other signaling events downstream of the calcium mobilization, the effect of the antibody on the PMA-stimulated respiratory burst was also investigated. The neutrophils were stimulated with PMA after the cells had been incubated with 200 ng/ml A6D for 60 min. Several simultaneous measurements of the PMA-stimulated respiratory bursts in the antibody-treated and control cells were performed using a photon counter equipped with a rotating sample holder. It was found that the kinetics of the PMA-stimulated respiratory burst in the antibody-treated cells did not differ from that in controlled cells, and the difference in intensity of the burst for the both cases was always within the measurement errors. A typical result is shown in Fig. 5. Almost no inhibition of the PMA-stimulated respiratory burst by the antibody was observed, which indicates that the antibody of PI4K does not interfere with the activation of protein kinase C by PMA.

*The Activity of PI4K in fMLP-stimulated Cells Treated with PAO or the PI4K Antibody*—Because the respiratory burst of neutrophils reaches its maximum at about 40 s after fMLP stimulation, the activities of the PI4K in the cells were measured 40 s after fMLP stimulation. According to the methods described under "Experimental Procedures," the activities of PI4K in the cells, which were preincubated with 200 nM, 600 nM, or 1 mM PAO for 5 min or with 1, 4, or 20 ng/ml A6D for 60 min, were measured 40 s later after stimulation. In the measurements, the cells preincubated with human IgG of corresponding concentrations were used as control. In addition, the PI4K activities in resting cells and in the fMLP-stimulated cells without PAO or A6D treatment were checked at the same time. The results are shown as a histogram in Fig. 6. It can be seen that either PAO or the PI4K antibody inhibits the activity of PI4K in fMLP-stimulated neutrophils in a concentration-dependent manner. It seems that PAO inhibits the fMLP-stimulated respiratory burst in neutrophils more effectively than the antibody. It was found that the antibody directed against type II PI4K could only inhibit the PI4K activity by about 40% even at the higher concentration of 20 ng/ml. The PI4K activity is higher in fMLP-stimulated cells than in resting cells, suggesting that the involvement of activation of PI4K in fMLP stimulation is in no doubt. The measurement of PI4K activity in the human IgG-treated cells showed that IgG does not inhibit PI4K activity in fMLP-stimulated neutrophils at all concentrations investigated. To know the full time course of PI4K activity with the antibody, the PI4K activities were also measured in the fMLP-stimulated cells that had been incubated with 20 ng/ml antibodies for 20, 40, and 60 min. The results showed that the PI4K activity in the fMLP-stimulated cells decreased as the preincubation increased. For comparison with the time-

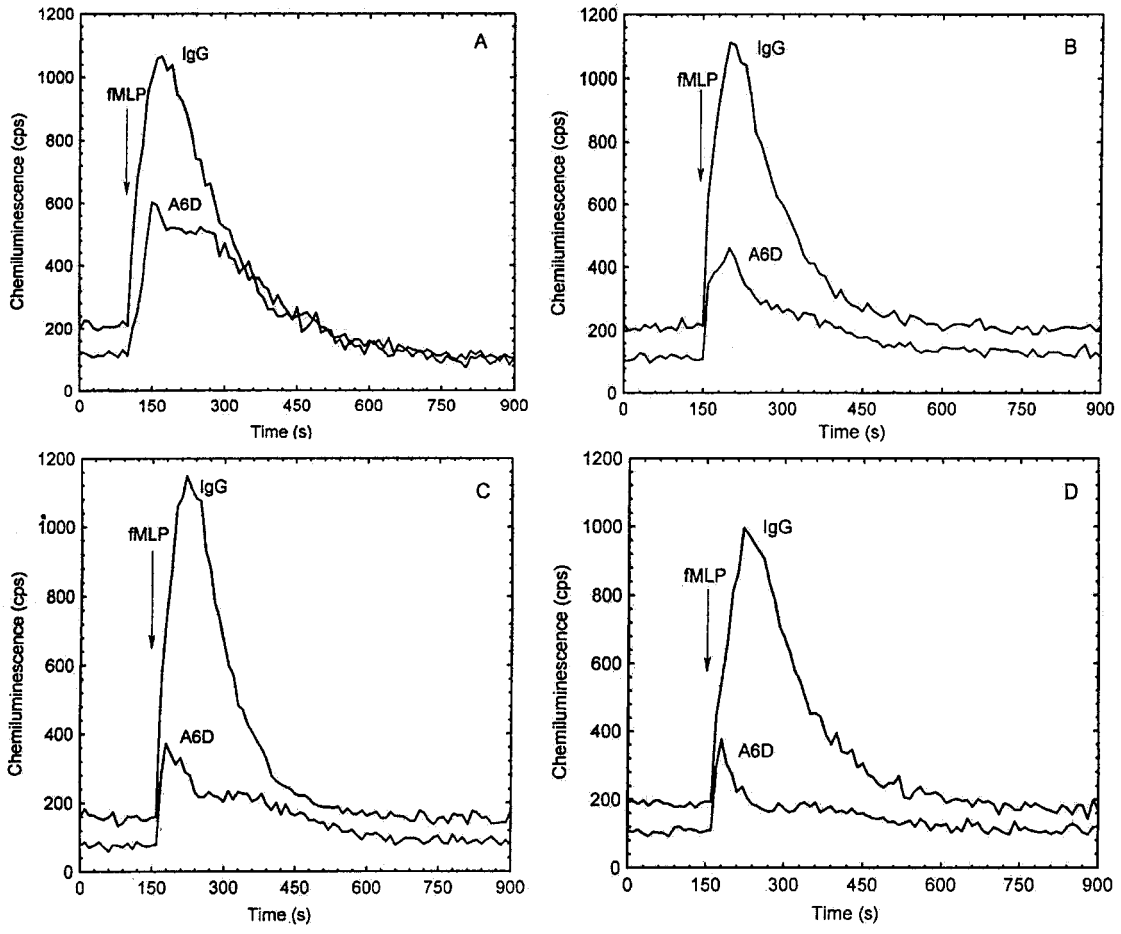


FIG. 3. **Inhibition of the respiratory burst by the PI4K antibody (A6D) of different concentrations in the fMLP-stimulated neutrophils.** The fMLP-stimulated respiratory burst in the cells preincubated with human IgG of the corresponding concentration was measured simultaneously as a control. $10^6$/ml cells were suspended in HBSS containing 1 $\mu$M luminol, and A6D and IgG of different concentrations were added to the cell suspension 60 min before 200 nM fMLP stimulation. The respiratory bursts in A6D- and IgG-treated cells were measured simultaneously as concomitant chemiluminescence at 37 °C. *Panel A*, 1 ng/ml A6D and IgG; *panel B*, 4 ng/ml A6D and IgG; *panel C*, 20 ng/ml A6D and IgG; *panel D*, 100 ng/ml A6D and IgG.

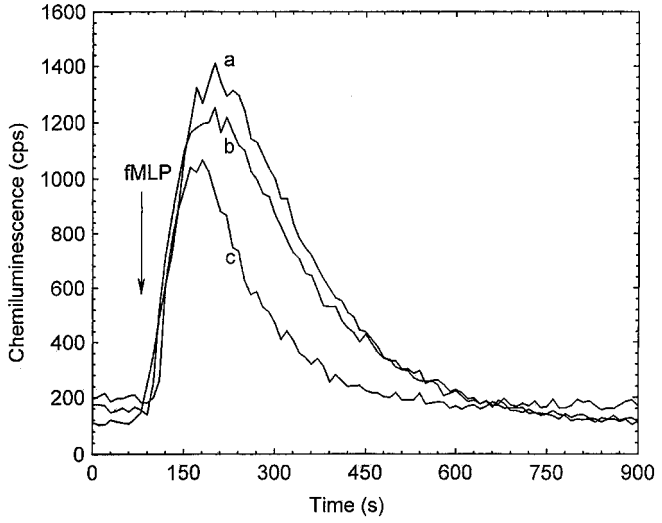


FIG. 4. **Inhibition of the fMLP-stimulated respiratory burst by human IgG of different concentrations in neutrophils.** $10^6$/ml cells were suspended in HBSS containing 1 $\mu$M luminol. No IgG (*curve a*), 20 ng/ml IgG (*curve b*), or 100 ng/ml IgG (*curve c*) was added to the cell suspension 60 min before 200 nM fMLP stimulation. The respiratory bursts were measured as concomitant chemiluminescence at 37 °C.

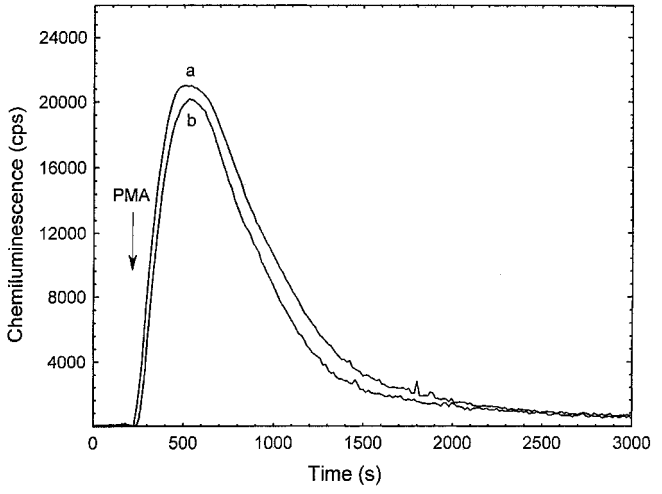


FIG. 5. **Effect of the PI4K antibody on the PMA-stimulated respiratory burst in neutrophils.** $1 \times 10^6$/ml cells were suspended in HBSS containing 1 $\mu$M luminol. No antibody (*curve a*) or 200 ng/ml antibody (*curve b*) was added to the cell suspension 60 min before 100 nM PMA stimulation. Respiratory bursts were measured as concomitant chemiluminescence at 37 °C.

dependent inhibition of the fMLP-stimulated respiratory burst by the antibody, this time course of PI4K activity with the antibody is also plotted as *curve b* in Fig. 2. It is interesting to note that the loss of PI4K activity caused by the antibody against the type II PI4K does not match the inhibition of the stimulated respiratory burst by the antibody. Less inactivation of PI4K and more inhibition of the cell response were observed.

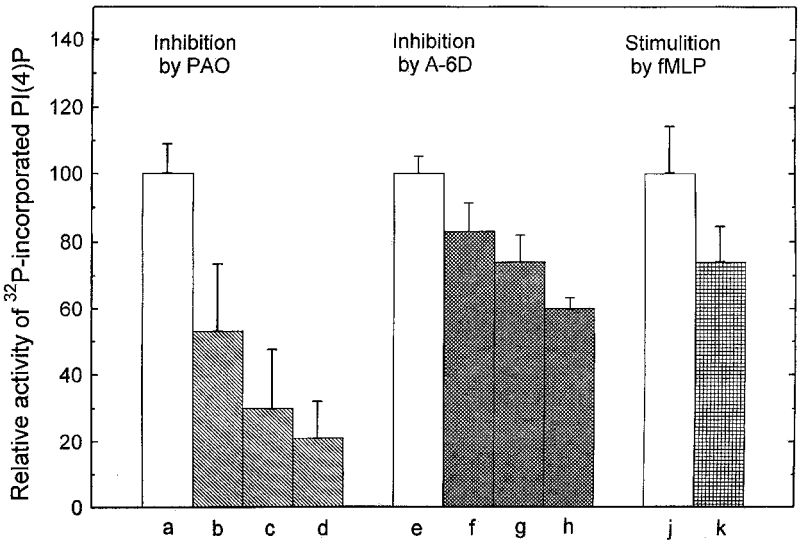


FIG. 6. **Relative activities of PI4K in the fMLP-stimulated neutrophils preincubated with PAO or the PI4K antibody A6D.** *Bar a*, no PAO; *b*, 200 nM PAO; *c*, 600 nM PAO; *d*, 1 mM PAO; *e*, no A6D; *f*, 1 ng/ml A6D; *g*, 4 ng/ml A6D; or *h*, 20 ng/ml A6D was added to the neutrophil suspension ($5 \times 10^6$ cells/ml, in HBSS at 37 °C). The cells were cooled quickly in an ice bath 40 s after the addition of 200 nM fMLP in each sample. The activities of PI4K in the cells were assayed as the activities of $^{32}$P-incorporated PI(4)P according to the procedure described under "Experimental Procedures." *Bars j* and *k* are the measured PI4K activities in fMLP-stimulated untreated cells and resting cells, respectively. All assays were performed in duplicate.

## DISCUSSION

Increasing evidence has accumulated to show that the macromolecules of molecular mass in the range of several tens to several hundred kDa can penetrate the cell surface and enter the cytosol by endocytosis. It has been reported that $^{125}$I-labeled bovine serum albumin (66 kDa) can be internalized into growing astrocytes in primary culture and return to the extracellular space after cycling (28). The proteins caveolin, clathrin, rab5, and β-COD were claimed by the authors to be involved in endocytosis. In addition, several investigators have reported the internalization of horseradish peroxidase (44 kDa) in NIHK 3025 cells (29) and goldfish intestinal epithelium (30). Similar to the antibody used in this study, the uptake of the IgG complex (150–200 kDa) by macrophages was also reported (24). In the present study, we showed a gradual accumulation of the fluorescence-labeled PI4K antibody in the cytosol of neutrophils after the antibody was added to the cell-containing buffer in terms of digital fluorescence microscopy. The fluorescence microscopic imaging of the cells clearly shows that the antibody does enter the cells from extracellular space with a slow time course. The time course seems consistent with the dependence of either the inhibition of the fMLP-stimulated respiratory burst or the activation of PI4K on the time that cells were preincubated with the antibody (see Fig. 2). The latter not only indicates that a certain period of time is needed for enough antibody to enter into cells, but also that the internalized antibody can function as an inhibitor for the respiratory burst and inactivator of PI4K in cells.

The measured effects of PAO and the PI4K antibody on both the respiratory burst and the activity of PI4K in fMLP-stimulated cells demonstrate that both of them inhibit the respiratory burst by inactivating PI4K. However, PAO inhibits not only the fMLP-stimulated respiratory burst, but also PMA (a direct activator of protein kinase C)-stimulated one, which indicates that PAO inhibits the respiratory burst not only by inhibiting PI4K but also by some other mechanism. In this regard, PAO may not be a specific inhibitor of PI4K. Fortunately, It was found that the antibody directed against type II PI4K inhibited the fMLP-stimulated respiratory burst but not the respiratory burst stimulated by PMA. Although some nonspecific antibody effect on the fMLP-stimulated respiratory burst was observed using the cells preincubated with human IgG as an irrelevant monoclonal antibody, the specificity of the monoclonal antibody in activating PI4K activity observed in this study seems reasonable and expectable. The PI4K activity measurements showed that only the PI4K antibody but not the IgG inhibited the activity of PI4K in the fMLP-stimulated neutrophils. The time course of PI4K activity with the antibody provides further evidence that more PI4Ks were inactivated when more PI4K antibodies were entered into cells. The assay of PI4K activities in fMLP-stimulated neutrophils showed that PAO seems more effective than the antibody in inactivating PI4K activity. This may because the used antibody can only inactivate the type II PI4K but not other isoforms of PI4K which may still provide some $PI(4,5)P_2$ for either PI3K or PLC to utilize.

It was interesting to notice that the inhibition of PI4K activity by the antibody was less than the inhibition of fMLP-stimulated respiratory burst by the antibody. For example, the antibody at a concentration of 20 ng/ml inhibited the cell response by about 70%, but PI4K activity was inhibited by only about 40%. This discrepancy may be because during the preincubation of cells with the antibody the $PIP_2$ and/or $PIP_3$ pools may be depleted to some extent by reduced replenishment of PI(4)P, then the inactivation of PI4K by the antibody and the depletion of existing $PIP_2$ and/or $PIP_3$ in the pools together result in an inhibition of the cell response. Thus, the reduced activity of PI4K at the moment of fMLP stimulation partially accounts for the inhibition of the stimulated respiratory burst in the cells, and the depletion of the $PIP_2$ and/or $PIP_3$ pools accounts for the response as another part. In this study, the maximal inhibition of the fMLP-stimulated respiratory burst by the PI4K antibody was found to be about 70%, and the maximal inactivation of PI4K activity was about 40% (see Figs. 2 and 3). It may be deduced that about 30% of the inhibition on the cell response by the antibody is possibly the result of the depletion in the $PIP_2$ and/or $PIP_3$ pools.

If we consider the PLC signaling and PI3K signaling as a whole, we may conclude that about 40% of the $PI(4,5)P_2$ is promptly converted from PI by type II PI4K and PIP5K activated in the fMLP-activated neutrophils.

This result may also suggest that the contribution of PI4K cannot be excluded from the role played by PI3K in signal transduction and that PI3K, PI4K, and PIP5K must be considered as an integrated family for the PI(3,4,5)$P_3$-initiated signaling. In addition, PI4K and PIP5K also play a role in PLC signaling, for which some further investigation may be needed.

*Acknowledgments*—We thank Prof. Fu-Yu Yang, the National Laboratory of Biomolecules, for encouragement in this study.

REFERENCES

1. Baggiolini, M., Dewald, B., and Moser, B. (1997) *Annu. Rev. Immunol.* **15,** 675–705
2. Locati, M., and Murphy, P. M. (1999) *Annu. Rev. Med.* **50,** 425–440
3. Wu, D., LaRosa, G. J., and Simon, M. I. (1993) *Science* **26,** 1101–1103
4. Stoyanov, B., Volinia, S., Hanck, T., Rubio, I., Loubtchenkov, M., Malek, D., Stoyanova, S., Vanhaesebroeck, B., Dhand, R., Nurnberg, B., Gierschik, P., Seedorf, K., Hsuan, J. J., Waterfield, M. D., and Wetzker, R. (1995) *Science* **269,** 690–693
5. Stephens, L. R., Eguinoa, A., Erdjument-Bromage, H., Lui, M., Cooke, F., Coadwell, J., Smrcka, A. S., Thelen, M., Cadwallader, K., Tempst, P., and Hawkins, P. T. (1997) *Cell* **89,** 105–114
6. Franke, T. F., Kaplan, D. R., and Cantley, L. C. (1997) *Cell* **88,** 435–437
7. Alessi, D. R., Kozlowski, M. T., Weng, Q. P., Morrice, N., and Avruch, J. (1998) *Curr. Biol.* **8,** 69–81
8. Fruman, D. A., Meyers, R. E., and Cantley, L. C. (1998) *Annu. Rev. Biochem.* **67,** 481–507
9. Traynor-Kaplan, A. E., Thompson, B. L., Harris, A. L., Taylor, P., Pmann, G. M., and Sklar, L. A. (1989) *J. Biol. Chem.* **264,** 15668–15673
10. Stephens, L. R., Hughes, K. T., and Irvine, R. F. (1991) *Nature* **351,** 33–39
11. Nishioka, H., Horiuchi, H., Arai, H., and Kita, T. (1998) *FEBS Lett.* **441,** 63–66
12. Condliffe, A. M., Hawkins, P. T., Stephens, L. R., Haslett, C., and Chilvers, E. D. (1998) *FEBS Lett*. **439,** 147–151
13. Gehrmann, T., and Heilmeyer, M. G. (1998) *Eur. J. Biochem.* **253,** 357–370
14. Nakanishi, S., Catt, K. J., and Balla, T. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 5317–5321
15. Meyers, R., and Cantley, L. C. (1997) *J. Biol. Chem.* **272,** 4384–4390
16. Downing, G. J., Stanley, K., Nakanishi, S., Catt, K. J., and Balla, T. (1996) *Biochemistry* **35,** 3587–3594
17. Reynolds, A. J., Heydon, K., Bartlett, S. E., and Hendry, I. A. (1999) *J. Neurochem.* **73,** 87–95
18. Wiedemann, C., Schafer, T., and Burger, M. M. (1996) *EMBO J.* **15,** 2094–2101
19. Sorensen, S. D., Liseman, D. A., McEwen, E. L., Heacock, A. M., and Fisher, S. K. (1998) *Mol. Pharmacol.* **53,** 827–836
20. Scholz, G., Barritt, G. J., and Kwok, F. (1991) *Eur. J. Biochem.* **201,** 249–255
21. Yue, J. C., and Gai, L. Y. (1999) *Prog. Biochem. Biophys.* **26,** 473–477 (in Chinese)
22. Endemann, G. C., Graziani, A., and Cantley, L. C. (1991) *Biochem. J.* **273,** 63–66
23. Yue, J. C., Ouyang, Y. S., and Guo, Z. Q. (2000) *Chin. J. Biochem. Mol. Biol.* **16,** 234–239 (in Chinese)
24. Mattana, J., Sankaran, R. T., and Singhal, P. C. (1995) *Am. J. Pathol.* **14,** 7529–7540
25. Payrastre, B., Nievers, M., Boonstra, J., Breton, M., Verkleij, A. J., and Van Bergen en Henegouwen, P. M. (1992) *J. Biol. Chem.* **267,** 5078–5084
26. Shore, J. D., Day, D. E., Francis-Chmura, A. M., Verhamme, I., Kvassman, J., Lawrence, D. A., and Ginsburg, D. (1995) *J. Biol. Chem.* **270,** 5395–5398
27. Rajarathnam, K., Hochman, J., Schindler, M., and Ferguson-Miller, S. (1989) *Biochemistry* **28,** 3168–3176
28. Megias, L., Guerri, C., Fornas, E., Azorin, I., Bendala, E., Sancho-Tello, M., Duran, J. M., Tomas, M., Gomez-Lechon, M. J., and Renau-Piqueras, J. (2000) *Int. J. Dev. Biol.* **44,** 209–221
29. Berg, K., Selbo, P. K., Prasmickaite, L., Tjelle, T. E., Sandvig, K., Moan, J., Gaudernack, G., Foodstad, O., Kjolsrud, S., Anholt, H., Rodal, G. H., Rodal, S. K., and Hogset, A. (1999) *Cancer Res*. **59,** 1180–1183
30. Kiliaan, A. J., Scholten, G., Bijlsma, P. B., Dekker, K., and Groot, J. A. (1996) *Cell Tissue Res.* **28,** 551–556