Cyclic AMP-increasing Agents Interfere with Chemoattractant-induced Respiratory Burst in Neutrophils as a Result of the Inhibition of Phosphatidylinositol 3-Kinase Rather Than Receptor-operated Ca\(^{2+}\) Influx*

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Mosleh U. Ahmed‡, Kaoru Hazeki‡, Osamu Hazeki§, Toshiaki Katada§, and Michio Ui*†

From the Ui Laboratory, the Institute of Physical and Chemical Research, Hirosawa 2-1, Wako-shi 351-01 and the §Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113, Japan

Superoxide anion and arachidonic acid were produced in guinea pig neutrophils in response to a chemoattractant peptide formyl-methionyl-leucyl-phenylalanine (fMLP). Both responses were markedly, but the former response to a phorbol ester was not at all, inhibited when the cellular cAMP level was raised by prostaglandin E\(_2\) combined with a cAMP phosphodiesterase inhibitor. Increasing cAMP was also inhibitory to fMLP-induced activation of phosphatidylinositol (PI) 3-kinase and Ca\(^{2+}\) influx without any effect on the cation mobilization from intracellular stores. The fMLP-induced respiratory burst was abolished when PI 3-kinase was inhibited by wortmannin or LY294002, but was not affected when Ca\(^{2+}\) influx was inhibited. On the contrary, fMLP released arachidonic acid from the cells treated with the PI 3-kinase inhibitors as well as from non-treated cells, but it did not show cellular Ca\(^{2+}\) uptake was prevented. The chemotactic peptide activated PI 3-kinase even in cells in which the receptor-mediated intracellular Ca\(^{2+}\) mobilization and respiratory burst were both abolished by exposure of the cells to a permeable Ca\(^{2+}\)-chelating agent. Thus, stimulation of fMLP receptors gave rise to dual effects, activation of PI 3-kinase and intracellular Ca\(^{2+}\) mobilization; both effects were necessary for the fMLP-induced respiratory burst. Increasing cellular cAMP inhibited the respiratory burst and arachidonic acid release as a result of the inhibitions of PI 3-kinase and Ca\(^{2+}\) influx, respectively, in fMLP-treated neutrophils.

The intracellular signaling pathways responsible for the formyl-methionyl-leucyl-phenylalanine (fMLP)\(^1\)-induced respiratory burst in phagocytes remain to be fully understood as yet, although the activation mechanism of the respiratory burst oxidase is currently elucidated as an assembly of a number of membrane-bound and cytosolic components, including gp91phox, p22phox, p47phox, p67phox, and Rac (see Ref. 1 for review).

In general, one of the best strategies for identification of essential cellular signals involved in a cell response to a receptor stimulation is to search for the target with which an inhibitor interacts to abolish the response efficiently. Pertussis toxin was a good inhibitor and was successfully applied to the fMLP-induced respiratory burst. Prior exposure of guinea pig neutrophils to low concentrations of pertussis toxin for several hours prevented the cells from producing superoxide anion in response to the subsequent addition of fMLP (2). The prevention resulted from the toxin-induced ADP-ribosylation of G protein (Gi-2), the chemical modification by which the modified G protein is uncoupled from receptors. Evidence has been thus provided for involvement of the G protein in the fMLP-induced respiratory burst via phospholipase C activation (2–4).

An additional example of useful inhibitors of the phagocytic respiratory burst is wortmannin, a fungal metabolite with hydrophobic sterol structure. Baggiolini and his colleagues (5, 6) first reported that incubation of human neutrophils with wortmannin or 17-hydroxywortmannin for 5 min inhibited fMLP-induced O\(_2\) generation and degranulation without affecting the chemoattractant-induced Ca\(^{2+}\) mobilization. The inhibition induced by wortmannin was so selective that it did not inhibit similar responses of neutrophils to a phorbol ester, a direct inhibitor of protein kinase C. Wortmannin has lately been proven to abolish the cellular signaling as a result of its direct interaction with phosphatidylinositol (PI) 3-kinase, providing convincing evidence for essential roles of this enzyme or the products of the enzyme in signaling pathways connecting G protein-coupled receptors and the respiratory burst (7, 8).

The purpose of the present paper is further application of this useful strategy to identification of important signaling pathways responsible for the fMLP-induced respiratory burst in guinea pig neutrophils. Increases in cAMP within cells have long been known to inhibit receptor-mediated respiratory bursts in reticulocytes and macrophages (e.g. Refs. 9 and 10 and references cited therein). We have identified intracellular signals that were impaired in parallel with the inhibition of fMLP-induced O\(_2\) generation upon the addition of cAMP-increasing agents to the cells.

EXPERIMENTAL PROCEDURES

Materials—Materials were obtained from the following sources: cAMP, Bt\(_2\)cAMP, fMLP, IBMX, PGE\(_1\), PMA, cytochrome c, A23187, staurosporine, and bovine serum albumin from Sigma; Fura2/AM and BAPTA/AM from Dojinkin Laboratories (Kumamoto, J japan); bisindolylma- leimide (GF109203X) from Calbiochem; \(^3\)P, and \(^32\)P]arachidonic acid from DuPont NEN. Wortmannin and pertussis toxin were kind gifts of

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† The first two authors contributed equally to this work.
‡ To whom correspondence should be addressed: the Ui Laboratory, the Institute of Physical and Chemical Research, Hirosawa 2-1, Wako-shi 351-01, J apan. Tel.: 81-48-462-1111 (ext. 3431); Fax: 81-48-462-4693.
§ The abbreviations used are: fMLP, formyl-methionyl-leucyl-phenylalanine; BAPTA, O,O′-bis(2-aminophenyl)ethylene glycol-N,N′,N″-tetraacetic acid; Bt\(_2\)cAMP, dibutyryl cAMP; (Ca\(^{2+}\)), intracellular free Ca\(^{2+}\) concentration; Fura2/AM, Fura2 acetoxymethyl ester; IBMX, 3-isobutyl-1-methyikanthine; PGE\(_1\), prostaglandin E\(_1\); PI, phosphatidylinositol; PMA, phorbol 12-myristate 13-acetate; PIP\(_2\), inositol 1,4,5-trisphosphate; MAP, mitogen-activated protein.
from Drs. Y. Matsuda (Kyowa Hakko Kogyo Co.) and M. Tamura (Kaken Seiyaku Co.), respectively. The stock solution (10 mM) of wortmannin was prepared in dimethyl sulfoxide, stored at −20 °C, and diluted with appropriate buffer immediately before use.

Cellisolated by density gradients were isolated from peripheral blood of men and women as described previously (11). The cells were washed twice with a solution containing 10 mM KCl, and 5.5 mM glucose. These labeled cells were resuspended at the density of 106 cells/ml in the regular Krebs-Ringer-Hepes medium (134 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2.5 mM CaCl2, 5 mM glucose, 0.2% bovine serum albumin, 20 mM Heps, pH 7.4). The cells were then resuspended with a rubber policeman, washed, and finally suspended in the regular Krebs-Ringer-Hepes medium to be subjected to further analyses. Freshly isolated (noncultured) cell suspensions were used in some experiments in which cells were not intended to be loaded with the tracer, the fluorescent dye, or the radioactive metabolite; essentially the same results were obtained between the fresh and cultured cells as to O2 generation.

Selective Inhibition of Respiratory Bursts by cAMP-increasing Agents and Various Inhibitors—Fig. 1 shows typical O2 generation by measurement of the reduction of cytochrome c based on the difference spectrum at 550–540 nm. Measurement of [Ca2+]i—The Fura2-loaded neutrophils (5 × 106 cells/ml), after being exposed to vehicles or inhibitors for 10 or 15 min at 37 °C, were analyzed with fMLP or PMA in the presence of 80 μM cytochrome c. The stimulation was stopped by adding ice-cold Ca2+-free Krebs-Ringer-Hepes medium (fortified with 10 mM EGTA), and the mixture was quickly centrifuged (1,500 g for 10 s) to afford the supernatant to be assayed for O2 generation by measurement of the ratio to O2 generation.

Arachidonic Acid Release—Aliquots (4 × 106 cells/0.5 ml) of [3H]arachidonate-labeled neutrophil suspensions, prepared as described above, were first exposed to inhibitors for 10 min and then incubated for 15 min with stimulators at 37 °C. The incubation was stopped by the addition of 0.5 ml of Ca2+-free Krebs-Ringer-Hepes medium with [3H]arachidonate. Fluorescence was determined in a dual excitation wavelength fluorimeter (Hitachi, F-2000) and expressed as a ratio of the fluorescence at the two excitation wavelengths to calculate [Ca2+]i according to the equation described previously (12).

[32P] Labeling of Phospholipids as a Measure of PI 3-Kinase in Intact Cells—Production of [32P]-labeled PIP2 was estimated by the method described previously (13). Neutrophil suspensions (106 cells/ml) were labeled with 32P-γ-ATP (150 μCi/ml) by 30-min incubation at 30 °C in the medium containing 10 ml Heps-NaOH, pH 7.4, 136 ml NaCl, 4.9 mM KCl, and 5.5 mM glucose. These labeled cells were washed twice, resuspended at the density of 107 cells/ml in the regular Krebs-Ringer-Hepes medium, and, as 250-μl aliquots, incubated at 37 °C for 10 or 15 min with or without inhibitors before the final stimulation with 0.1 μM fMLP for 15 or longer. After stimulation, cells were lysed by vigorous stirring in 1.55 ml of chloroform/methanol/8% HClO4 (50:100:5), followed by addition with 1 ml of the chloroform:H2O (1:1) mixture to separate the organic phase, which was washed with chloroform-saturated 1% HClO4 and dried in vacuo. The lipids were dissolved in 20 μl of chloroform/methanol (95:5) and spotted on a thin-layer plate (Silica Gel 60, Merck), which had been impregnated with potassium oxalate by the procedure of development in a solvent system of 1.2% potassium oxalate-containing methanol/water (2:3) and activated by heating at 110 °C for 20 min before spotting. The plate was developed in chloroform/methanol/acetic acid/water (80:30:26:24:14), dried, and visualized for the radioactivities with a Fuji BAS2000 Bioimaging Analyzer.

Measurement of CAMP—Incubation of neutrophil suspensions with 0.3 mM IBMX was stopped by the addition of HCl to make the final concentration of 0.1 N, which was followed by maintaining the dishes in boiling water for 3 min and centrifugation. The resultant supernatant was submitted for radioimmunossay of cAMP as described previously (11).

RESULTS

Selective Inhibition of Respiratory Bursts by cAMP-increasing Agents and Various Inhibitors—Fig. 1 shows typical O2 generation responses of guinea pig neutrophils to additions of increasing concentrations of fMLP and PMA. The concentration-dependent increases in O2 generation were observed for fMLP and PMA with the maximal responses being at 100 and 10 nM, respectively. The cell responses to fMLP were markedly reduced (Fig. 1A), but those to PMA were not affected (Fig. 1B), when the cells had been exposed for 10 min to agents that can increase the cellular content of cAMP (an adenylate cyclase activator, PGE1, an inhibitor of cAMP-hydrolyzing phosphodiesterase, IBMX, and their combination) or a cell-permeable cAMP analogue (Bt2cAMP). Actually, the cAMP content in neutrophils was less than 1, 6.2 ± 0.1, and 91 ± 4 pmol/106 cells (with the number of observations of six) after 10-min incubation without any addition, with 0.3 mM IBMX alone and its combination with 10 μM PGE1, respectively, under these conditions. Prior exposure of neutrophils to pertussis toxin also abolished the cell response to fMLP without affecting the response to PMA (data not shown).

The respiratory burst induced by fMLP was likewise inhibited by wortmannin (Fig. 2A) and staurosporine (Fig. 2B) in concentration-dependent manners. LY294002, a wortmannin-like inhibitor of PI 3-kinase (14, 15), gave rise to effects, just like wortmannin (8), very similar to those caused by cAMP-increasing agents; fMLP-induced O2 generation was, but the PMA-induced generation was not at all, inhibited by the inhibitor (Fig. 2C). It is conceivable that the increase in cAMP may interact with the step that functions, like PI 3-kinase, upstream of protein kinase C in G protein-initiated signaling pathways. Bisindolylmaleimide, an inhibitor of protein kinase C much more specific than staurosporine (16), inhibited, in concentration-dependent manners, the fMLP-induced respiratory burst as well as PMA-induced one (Fig. 2D). Thus, activation of protein kinase C, which was not susceptible to cAMP increases within cells or PI 3-kinase inhibitors such as wortmannin and LY294002, was essential for the G protein-initiated respiratory burst. In fact, staurosporine did, but wortmannin did not, inhibit PMA-induced O2 generation at all the concentrations of the phosphor ester employed (Fig. 2E).

Inhibition by cAMP-increasing Agents of Receptor-operated...
Ca²⁺ Influx—Fura2-loaded neutrophils exhibited marked increases in [Ca²⁺] in response to the addition of fMLP (Fig. 3). The increase in [Ca²⁺], in response to fMLP was transient. The rapid increase was followed by a rapid decrease to form a sharp peak of [Ca²⁺], within 30–60 s, after which [Ca²⁺] leveled off at a much higher level than the prestimulation value in Ca²⁺-containing medium (Fig. 3A). Such sustained increases in [Ca²⁺], were mostly due to continuous Ca²⁺ influx following receptor stimulation; the rapid [Ca²⁺] peak became much smaller in magnitude in the incubation medium from which Ca²⁺ was nominally omitted (Fig. 3B). Addition of Ca²⁺, within 3 min after receptor stimulation, into the Ca²⁺-free medium caused a marked increase in [Ca²⁺] (Fig. 3B). The Ca²⁺ addition to the cell suspension in the Ca²⁺-free medium, without receptor stimulation, did not cause any significant increase in [Ca²⁺], (data not shown, but see a dotted trace in Fig. 4 for example). Thus, the first and the second peaks of [Ca²⁺], in Fig. 3B reflect mobilization of Ca²⁺ from the intracellular stores and Ca²⁺ influx from the exterior, respectively, in response of neutrophils to fMLP receptor stimulation.

When fMLP receptors were uncoupled from G proteins by prior exposure of cells to pertussis toxin, the agonist failed to increase [Ca²⁺], significantly in either the presence (Fig. 3C) or the absence (Fig. 3D) of extracellular Ca²⁺. No significant [Ca²⁺], increase occurred upon further addition of Ca²⁺ into the Ca²⁺-free medium bathing pertussis toxin- and fMLP-treated neutrophils, providing evidence for involvement of the toxin-sensitive G protein in the Ca²⁺ influx (Fig. 3D). In the cells pre-exposed to the cAMP-increasing agents (PGE₁ plus IBMX), the peak value of fMLP-induced [Ca²⁺], increase was slightly smaller than the non-exposed cells and was followed by the return to the level much lower than that observed for control (Fig. 3E as compared with 3A), suggesting that receptor-coupled Ca²⁺ influx was inhibited upon increases in the cellular Ca²⁺ concentration. In fact, addition of fMLP into Ca²⁺-free medium caused the same increase in [Ca²⁺], in the cells treated with cAMP-increasing agents as in control cells, whereas the subsequent addition of Ca²⁺ elicited much smaller increase in [Ca²⁺], in the former cells than in the latter cells (Fig. 3F). Thus, elevation of intracellular cAMP attenuated fMLP receptor-operated Ca²⁺ influx without affecting the receptor-mediated mobilization of Ca²⁺ from the internal stores. There was only 20–30% decrease in fMLP-induced generation of inositol 1,4,5-trisphosphate, when guinea pig neutrophils had been exposed to the cAMP-generating agents (data not shown). Conceivably, such slightly attenuated generation of inositol 1,4,5-trisphosphate is still enough for the maximal mobilization of Ca²⁺ from the stores.

Ca²⁺ influx in Fura2-loaded neutrophils. Fresh guinea pig neutrophils were first exposed for 10 min to increasing concentrations of wortmannin (A), staurosporine (B), LY294002 (C), or bisindolylmaleimide (D) before further 15-min incubation with 0.1 μM fMLP (●), 0.1 μM PMA (▲) or without addition (○). In E, the first exposure was done to 0.1 μM wortmannin (●), 0.1 μM staurosporine (▲) or vehicle (○), and the further incubation was with increasing concentrations of PMA. These cells were then assayed for O₂ generation as described under "Experimental Procedures." The same results were reproduced in additional two or three experiments.

The inhibition of receptor-operated Ca²⁺ influx was very unique to the action of cAMP-generating agents, in the sense that other inhibitors of fMLP-induced superoxide anion release, such as wortmannin and staurosporine, did not inhibit but rather enhanced the receptor-coupled Ca²⁺ influx (Figs. 3,
G-J). Stauroporine may enhance Ca\(^{2+}\) entry by antagonizing protein kinase C-induced phosphorylation of proteins that is possibly involved in inhibition of capacitative Ca\(^{2+}\) influx in this cell type (17), although the mechanism for wortmannin-induced enhancement (Fig. 3H) remains to be clarified.

The addition of Ca\(^{2+}\) following thapsigargin into neutrophil suspensions in the Ca\(^{2+}\)-free medium gave rise to a marked increase in [Ca\(^{2+}\)]\(_i\), despite lack of the increase with preaddition of the endoplasmic Ca\(^{2+}\) pump inhibitor, due to the capacitated Ca\(^{2+}\) entry following emptying of the intracellular stores but not mediated by receptor stimulation (Fig. 4). This capacitated Ca\(^{2+}\) entry was not affected significantly, or inhibited only slightly, by exposure of cells to cAMP-generating agents (Fig. 4). Emptying the Ca\(^{2+}\) stores after the thapsigargin treatment was evidenced by the failure of the treated cells to respond to fMLP by increasing [Ca\(^{2+}\)]\(_i\) (data not shown). Thus, the major target of increased cAMP or cAMP-dependent protein kinase appears to be the protein(s) involved in receptor-operated, rather than store-operated, Ca\(^{2+}\) channels.

Ca\(^{2+}\) influx as a Mediator of Arachidonic Acid Release Rather than O\(_2\) \(_{\text{g}}\) Generation—The chemotactant-induced superoxide anion release was associated with an increase in Ca\(^{2+}\) influx (Fig. 3). Both effects of fMLP were markedly attenuated upon increases in intracellular CAMP. The increase in fMLP receptor-coupled entry of extracellular Ca\(^{2+}\), however, never played any essential role in O\(_2\) \(_{\text{g}}\) generation, which occurred and was inhibited by cAMP-increasing agents in the same magnitude in either the presence or absence of Ca\(^{2+}\) in the incubation medium (Fig. 5). In sharp contrast, arachidonic acid release provoked by fMLP receptor stimulation was highly dependent on the translocation of Ca\(^{2+}\) from the extracellular fluids (Fig. 6). No significant release of arachidonate was observed if the medium was depleted of Ca\(^{2+}\) (Fig. 6B).

Again, wortmannin and cAMP-increasing agents exerted different effects on the response of neutrophils. The fungal metabolite did not inhibit arachidonic acid release under any condition tested, whereas PGE\(_1\) plus IBMX was an inhibitor of the fMLP-induced release as strong as Ni\(^{2+}\) or Co\(^{2+}\), antagonists of transmembrane Ca\(^{2+}\) inflow (Fig. 6A). Ni\(^{2+}\) has been reported to be an inhibitor of receptor-operated Ca\(^{2+}\) entry (18). Stauroporine, like wortmannin, did not inhibit fMLP-induced arachidonic acid release. Failure of cAMP-increasing agents to inhibit Ca\(^{2+}\) ionophore-induced arachidonate production (Fig. 6A) is in agreement with the view that elevation of intracellular cAMP inhibits the fatty acid production as a result of the inhibition of receptor-coupled Ca\(^{2+}\) entry.

Prevention of fMLP-induced PI 3-Kinase Activation in Cells Whose CAMP Content Was Raised—\(^{32}\)P-Loaded neutrophils were incubated with fMLP for a short time of 15 s to detect fMLP-induced PIP\(_3\) production as a result of PI 3-kinase activation (Fig. 7A). Progressively less activation of PI 3-kinase was evoked by fMLP as the concentration of PGE\(_1\) was increased from 0.01 to 10 \(\mu\)M in the IBMX-containing medium bathing the cells for 10 min prior to the addition of the chemotactant. The range of the effective concentrations of PGE\(_1\) was essentially the same when comparison was made between PI 3-kinase activation (Fig. 7A) and O\(_2\) \(_{\text{g}}\) generation.
Inhibition of Phosphatidylinositol 3-Kinase by cAMP

**Fig. 7. fMLP-Induced PIP<sub>3</sub> Production and O<sub>2</sub><sup>−</sup> Generation in Neutrophils and Its Progressive Inhibition by Increasing Concentrations of PGE<sub>1</sub> in the Presence of IBMX.** A, 32P-labeled neutrophils were first incubated with increasing concentrations of PGE<sub>1</sub> for 10 min in the presence (+) or absence (−) of 0.3 mM IBMX and then incubated for 15 s with 0.1 μM fMLP or vehicle (None), as shown at the bottom, before being subjected to thin-layer chromatographic separation of phospholipids as described under "Experimental Procedures." The incubations were done in the regular Krebs-Ringer-Hepes medium and the autoradiogram of the thin-layer plate is shown, in which the position of spots corresponding to PIP<sub>3</sub> is indicated on the left. B, fresh neutrophils were first incubated with increasing concentrations of PGE<sub>1</sub> in the presence of 0.3 mM IBMX and then stimulated by 0.1 μM fMLP (●) or not (○) under the same conditions as used for A, except for the stimulation time of 15 min instead of 15 s. Superoxide anion production was determined, as described under "Experimental Procedures," and plotted as a function of PGE<sub>1</sub> concentrations. The cellular content of cAMP after the 15-min incubation is also shown (△). The results were reproduced in two or three separate experiments (Fig. 7B) as induced by fMLP. Moreover, the progressive inhibition of fMLP-induced O<sub>2</sub><sup>−</sup> generation depending on increasing concentrations of PGE<sub>1</sub> was inversely correlated with progressive increases of intracellular cAMP under the same conditions (Fig. 7B). Thus, the increase in intracellular cAMP antagonized both actions of fMLP to induce superoxide generation and to activate PI 3-kinase.

**PI 3-Kinase Activation and Intracellular Ca<sup>2+</sup> Mobilization Are Differentially Responsible for Superoxide Anion Generation—No increase in [Ca<sup>2+</sup>], was observed, even in the presence of the cation in the incubation medium, following fMLP addition to neutrophils that had been exposed to BAPTA/AM, a cell-permeable Ca<sup>2+</sup>-chelating agent (Fig. 8, A and B). The intracellular Ca<sup>2+</sup> chelation abolished the superoxide-generating response of the cells to fMLP as well (Fig. 8D), but did not impair the chemoattractant-induced activation of PI 3-kinase (Fig. 8C). Such a manner in which BAPTA/AM inhibited fMLP-induced O<sub>2</sub> generation formed a sharp contrast with the manner for the inhibition by cAMP-generating agents (Fig. 8D), which interfered with fMLP-induced PI 3-kinase activation under similar conditions (Fig. 8C) without affecting intracellular Ca<sup>2+</sup> mobilization (Fig. 3F). Thus, the mobilization of Ca<sup>2+</sup> from internal stores and the PI 3-kinase activation occurred in two separate signaling pathways emanating from fMLP receptor stimulation, eventually contributing to the same response of superoxide anion release from neutrophils. No arachidonic acid was released in response to fMLP from BAPTA-treated cells (data not shown), presumably because extracellular Ca<sup>2+</sup> did not enter the cells under these conditions (Fig. 8B).

**DISCUSSION**

Different Signals Arising from fMLP Receptor Stimulation Are Responsible for Superoxide Generation and Arachidonate Release—In the present paper, we measured two kinds of neutrophil responses, O<sub>2</sub>− generation and arachidonate acid release, to the addition of fMLP. Both responses were markedly attenuated by the agents (e.g. PGE<sub>1</sub> plus IBMX) causing accumulation of cAMP in cells. The other inhibitor of both responses was BAPTA/AM, a cell-permeable Ca<sup>2+</sup>-chelating agent, that abolished fMLP-induced intracellular Ca<sup>2+</sup> mobilization and the subsequent Ca<sup>2+</sup>-influx (Fig. 8). It is also well known that prior exposure of neutrophils to pertussis toxin abolishes all the cell responses to fMLP including O<sub>2</sub>− generation and arachidonate acid release (see Ref. 19 for review). Other inhibitors (or maneuvers of cells) tested here inhibited only either of these two responses. The chemoattractant-induced O<sub>2</sub>− generation was totally inhibited by protein kinase C inhibitors, staurosporine (and by bisindolylmaleimide also), and by direct and selective inhibitors of PI 3-kinase, wortmannin (and by LY294002 also), despite the failure of these inhibitors to affect fMLP-induced arachidonate acid release (Fig. 6). Vice versa, omission of Ca<sup>2+</sup> from the incubation medium, just like the addition into the medium of Ni<sup>2+</sup> or Co<sup>2+</sup>, direct inhibitors of transmembrane Ca<sup>2+</sup>-permeation, abolished arachidonate-releasing responses of neutrophils to fMLP and A23187, a Ca<sup>2+</sup>-ionophore (Fig. 6B) without any modification of fMLP-induced O<sub>2</sub>− generation and its susceptibility to cAMP-increasing agent-induced inhibition (Fig. 5).

It is very likely, therefore, that fMLP receptor stimulation triggered two signaling pathways each, with different susceptibility to certain inhibitors, separately leading to superoxide-generating arachidonate-producing responses of neutrophils. fMLP is one of the chemokine families whose receptors possessing seven membrane-spanning regions release, upon being occupied by the ligand, the G<sub>βγ</sub>-subunits from coupled pertussis toxin-sensitive G proteins. The signaling pathways may bifurcate at a point distal to the G protein, as evidenced by the fact that both responses are totally inhibited by treatment of the cells with pertussis toxin.

Ca<sup>2+</sup>-influx as cAMP-susceptible Signals Essential for fMLP-induced Arachidonic Acid Release—The G<sub>βγ</sub>-protein is a direct activator of phospholipase Cβ (20), a product of which, inositol 1,4,5-trisphosphate, causes mobilization of Ca<sup>2+</sup> from the internal stores in the endoplasmic reticulum that is immediately followed by external Ca<sup>2+</sup>-influx across the plasma membrane through the receptor-operated and store-operated channels. The Ca<sup>2+</sup>-influx was essential for the observed arachidonate release, which was abolished by omitting Ca<sup>2+</sup>-from the medium or by inhibiting the flux by Co<sup>2+</sup> or Ni<sup>2+</sup> (Fig. 6). Stimulation of Ca<sup>2+</sup>-influx by a Ca<sup>2+</sup>-ionophore gave rise to arachidonic acid release. Cytosolic phospholipase A<sub>2</sub> is likely to be activated as a result of Ca<sup>2+</sup>-dependent translocation into membranes where the substrate phospholipids are available (21–24).

Thus, it is very reasonable to assume that cAMP-increasing agents inhibit fMLP-induced arachidonate acid release as a result of their unique action to suppress Ca<sup>2+</sup>-influx following the mobilization of Ca<sup>2+</sup>-from internal stores (Fig. 3F), in accordance with previous reports (9, 10). It is tempting to speculate that receptor-operated Ca<sup>2+</sup>-channel is one of the targets of cAMP-dependent protein kinase, since cAMP-increasing agents were incapable of inhibiting thapsigargin-induced Ca<sup>2+</sup>-influx (Fig. 4) as well as Ca<sup>2+</sup>-ionophore-induced arachidonate acid release (Fig. 6A). Cyclic AMP has recently been reported to inhibit thrombin receptor-operated Ca<sup>2+</sup>-influx into platelets directly (25) or indirectly (26). Esential Role of cAMP-susceptible PI 3-Kinase in the fMLP-induced Respiratory Burst—The respiratory burst was inhibited by both inhibitors of PI 3-kinase and protein kinase C when it was induced by fMLP, but was not inhibited by the lipid kinase inhibitors when it was evoked by PMA (Fig. 2).
Inhibition of Phosphatidylinositol 3-kinase by cAMP

Figure 8. Differential effects of two respiratory burst inhibitors, an intracellular Ca\(^{2+}\)-chelating agent and a cAMP-increasing agent, on fMLP-induced PI 3-kinase activation. Guinea pig neutrophils, Fura2-loaded (A and B), \(^{32}\)P-labeled (C), or nontreated (D), had been exposed for 15 min to 0.3 mM IBMX plus 10 \(\mu\)M PGE\(_1\) or 20 \(\mu\)M BAPTA/AM as described in these panels before further incubation with or without 0.1 \(\mu\)M fMLP in the Ca\(^{2+}\)-containing regular medium. Tracings of [Ca\(^{2+}\)]\(_i\), with the addition of fMLP as indicated by arrows, are shown in A and B. The incorporation of \(^{32}\)P into the PI\(_3\) spots on the thin-layer plate during 15-s incubation with (hatched columns) or without (open columns) fMLP was determined as in Fig. 7 and is illustrated in C. \(O_2\) generation during 15-min incubation with or without fMLP is likewise shown in D. Typical data are taken from experiments repeated twice with essentially the same results.

Likewise, cAMP-increasing agents, which inhibit fMLP-induced PI 3-kinase and \(O_2\) generation, exerted no effect on the PMA-induced respiratory burst (Fig. 1). Phosphorylation of cytosolic components of the respiratory burst oxidase, particularly p47\(_{phox}\), is one of the important activation mechanisms of the oxidase (1). At least 7 of the 11 serine residues on the p47\(_{phox}\) molecule responsible for the activation were phosphorylated in PMA-activated neutrophils (27), and the phosphorylation maintains the oxidase in the assembled/active state (28). Thus, PI 3-kinase, which is inhibited when cellular cAMP increased, is located upstream of protein kinase C in the signaling cascade arising from RLP receptors and leading to the respiratory burst.

Priming Role of Ca\(^{2+}\) Mobilization for the fMLP-induced Respiratory Burst—A number of recent reports have revealed that stimulation of RLP receptors in neutrophils or neutrophil-type culture cell lines gives rise to phosphorylation of protein tyrosine residues (29–31), PI 3-kinase activation (7, 8, 13, 32–37), and Ras activation leading to MAP kinase cascades (38, 39). There are few papers reporting a decisive role of Ca\(^{2+}\) mobilization to trigger any tyrosine kinase signaling system. For instance, concanavalin A can activate a tyrosine kinase (40) or MAP kinase (38, 41) in Ca\(^{2+}\)-depleted human neutrophils. Likewise, an increase in \([\text{Ca}^{2+}]_i\) was not essential for fMLP-induced MAP kinase cascade activation (39). In fact, fMLP activated PI 3-kinase even in cells in which the chemoattractant did not cause Ca\(^{2+}\) mobilization, Ca\(^{2+}\) influx, or \(O_2\) generation after the treatment of cells with BAPTA/AM (Fig. 8C).

Thus, a readily achievable possibility is that internal Ca\(^{2+}\) mobilization primes, permits, or supports the signaling pathways responsible for \(O_2\) generation. In other words, the Ca\(^{2+}\) mobilization and the tyrosine kinase-related signalings including activation of PI 3-kinase are both indispensable for fMLP to provoke \(O_2\) generation. The small amount of Ca\(^{2+}\) mobilized in the Ca\(^{2+}\)-free medium (e.g. Fig. 3B) would be enough to support the respiratory burst.

The Site With Which an Increase in Cellular cAMP or cAMP-Dependent Protein Kinase Interacts—There is no evidence for direct phosphorylation by cAMP-dependent protein kinase (protein kinase A) of the p85 regulatory subunit or the p110 catalytic subunit of PI 3-kinase. Instead, a signaling pathway connecting the fMLP receptor stimulation and the PI 3-kinase activation appears to be a target of protein kinase A. Increasing cAMP or activation of cAMP-dependent protein kinase A has been reported to antagonize various receptor-initiated activation of MAP kinase cascades (42, 45–53). The target of protein kinase A was located upstream of MAP kinase (45), between p21\(^^{ras}\) and Raf-1 (47, 51) or Raf-1 itself (42, 48–50, 52). On the other hand, PI 3-kinase is directly activated by G\(_{bg}\) in neutrophils (54) and platelets (55) or by Ras (56), Rac (57), or Rho (58, 59) proteins in various cell types. Alternatively, PI 3-kinase has been found to be located upstream of the Ras protein in certain growth factor-initiated signaling pathways (60–62). Further studies are thus required before any decisive conclusion will be made as to how increasing cAMP inhibits fMLP-induced PI 3-kinase in neutrophils.
Inhibition of Phosphatidylinositol 3-Kinase by cAMP

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