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Comparison of tBuBHQ with chemotactic peptide and phorbol ester in O_2^- production in HL-60 cells

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Abstract

The effect of 2,5-di-(tert-butyl)-1,4-benzohydroquinone (tBuBHQ), a Ca^{2+} pump inhibitor, on superoxide anion (O_2^-) production was examined with a special reference to Ca^{2+} in HL-60 cells differentiated by dibutyryl cAMP, and compared with the effect of *N*-formyl-Met-Leu-Phe (fMLP) and phorbol 12-myristate 13-acetate (PMA). tBuBHQ caused O_2^- production and Ca^{2+} mobilization, but not phosphoinositide hydrolysis. fMLP caused O_2^- production, Ca^{2+} mobilization and phosphoinositide hydrolysis. PMA caused O_2^- production without affecting Ca^{2+} mobilization and phosphoinositide hydrolysis. EGTA and *O,O'*-bis(2-aminophenyl)ethyleneglycol-*N,N,N',N'*-tetraacetic acid, tetraacetoxymethyl ester (BAPTA/AM), an intracellular Ca^{2+} chelator, inhibited O_2^- production induced by fMLP, but not by tBuBHQ. Thapsigargin, another Ca^{2+} pump inhibitor, had a weak ability to produce O_2^-. fMLP, but not tBuBHQ, caused BAPTA/AM-sensitive activation of phospholipase A_2 and D. tBuBHQ caused O_2^- production by interacting with phosphatidylcholine in a cell-free system. The results suggest that tBuBHQ causes O_2^- production independent of Ca^{2+}, and Ca^{2+} might be a cofactor in the activation of phospholipase A_2 and D upstream in fMLP-induced O_2^- production.

Keywords: tBuBHQ (2,5-di-(tert-butyl)-1,4-benzohydroquinone); *N*-Formyl-Met-Leu-Phe; Phorbol ester; Superoxide anion; Ca^{2+}; BAPTA/AM (*O,O'*-bis(2-aminophenyl)ethyleneglycol-*N,N,N',N'*-tetraacetic acid, tetraacetoxymethyl ester)

1. Introduction

Superoxide anion (O_2^-) production is an important and well-known function of neutrophils against bacterial infections or inflammations. Individuals who have phagocytic cells that cannot produce O_2^- are susceptible to severe recurrent infections, known as chronic granulomatous disease (Smith and Curnutte, 1991). NADPH oxidase that converts O_2 to O_2^- is composed of cytochrome b_{558} existing in the membrane and two cytosolic factors, $p47^{phox}$ and $p67^{phox}$ (Finan et al., 1994). In addition, a small GTP-binding protein, Rac, also participates in this complex (Benna et al., 1994; Diekmann et al., 1994). When NADPH oxidase is activated, these factors assemble in the membrane (Abo et al., 1992; Quinn et al., 1993). Although the development of the cell-free oxydase system allows the understanding of the molecular mechanisms of O_2^- production, the signaling pathway of O_2^- production is not fully understood.

Chemotactic peptide, *N*-formyl-Met-Leu-Phe (fMLP) binds to a specific seven transmembrane receptor and activates phosphoinositide-specific phospholipase C mediated through pertussis toxin-sensitive heterotrimeric G protein in neutrophil-like differentiated HL-60 cells (Kikuchi et al., 1986). Activated phosphoinositide-specific phospholipase C hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to second messengers, inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol. IP_3 mobilizes Ca^{2+} from the intracellular Ca^{2+} store (Berridge and Irvine, 1984) and 1,2-diacylglycerol activates protein kinase C (Nishizuka, 1984). fMLP also activates phospholipase D (Xie et al., 1991; Gelas et al., 1992) and phospholipase A_2 (Okajima and Ui, 1984; Xing et al., 1994). Recent lines of evidence show that metabolites of these enzymes, phosphatidic acid and arachidonic acid, are necessary for an activation of NADPH oxidase in a cell-free system (Chuang et al., 1993; Ligeti et al., 1993; Qualliotine-Mann et al., 1993).

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Phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, is known to induce O_2^- production (Curnutte et al., 1994). Activation of protein kinase C results in phosphorylation of $p47^{phox}$ (Volpp et al., 1989) that contributes to O_2^- production. PMA is also known to activate phospholipase D (Billah et al., 1989). On the other hand, 2,5-tert-butyl-1,4-benzohydroquinone (tBuBHQ) is an inhibitor of the Ca^{2+} pump in the intracellular Ca^{2+} store (Moore et al., 1987; Kass et al., 1989; Furukawa et al., 1991), leading to Ca^{2+} influx and increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). tBuBHQ as well as thapsigargin, another Ca^{2+} pump inhibitor (Lytton et al., 1991), is used for examining the role of intracellular Ca^{2+}. Ca^{2+} pump inhibitors have an advantage for investigating Ca^{2+}-dependent process without affecting other signaling pathways. However, it remains unknown whether tBuBHQ affects O_2^- production.

O,O'-Bis(2-aminophenyl)ethyleneglycol-*N,N,N',N'*-tetraacetic acid, tetraacetoxymethyl ester (BAPTA/AM) is a useful tool for investigating the role of intracellular Ca^{2+}, because BAPTA/AM is hydrolyzed by esterase in the cells to BAPTA that can bind to free Ca^{2+} ions with high affinity. We can thus investigate the cell function under low $[Ca^{2+}]_i$ using BAPTA/AM.

In the present study, we investigated a role of Ca^{2+} in O_2^- production using three stimuli of tBuBHQ, fMLP and PMA. The results obtained suggest that tBuBHQ causes O_2^- production with a Ca^{2+}-independent mechanism, in spite of increasing $[Ca^{2+}]_i$. Ca^{2+} might be a cofactor for activation of phospholipase A_2 and phospholipase D upstream of protein kinase C activation and O_2^- production.

2. Materials and methods

2.1. Cell culture and differentiation

HL-60 cells were grown in RPMI 1640 containing 10% fetal bovine serum in a 37°C humidified incubator in an atmosphere of 95% air and 5% CO_2. Differentiation towards neutrophil-like cells was induced by culture in RPMI 1640 containing 0.5 mM dibutyryl cAMP, 5 μg/ml transferrin, 5 μg/ml insulin, 0.5 ng/ml sodium selenite and 25 mM Hepes for 72 h (Xie et al., 1991).

2.2. Measurement of superoxide anion production

O_2^- production was determined using superoxide dismutase-inhibitable cytochrome *c* reduction by measuring absorbance of cytochrome *c* at 549 nm and at 540 nm with a spectrophotometer (Hitachi, U-2000). Differentiated HL-60 cells were washed three times with Hanks' solution (composition in mM: NaCl 136.9, KCl 5.4, KH_2PO_4 0.44, NaH_2PO_4 0.17, $CaCl_2$ 1.2, $MgCl_2$ 0.49, $MgSO_4$ 0.41, $NaHCO_3$ 4.2, glucose 5.6, Hepes 10.0, EDTA 0.1, pH 7.4, and bovine serum albumin 1 mg/ml). The reactions were carried out at 37°C in glass tubes containing 2.5×10^6 cells in Hanks' solution containing 50 μM cytochrome *c* with or without 30 μg/ml superoxide dismutase. The reactions were stopped by the addition of *N*-ethylmaleimide to make a final concentration of 1 mM. The samples were centrifuged at $630 \times g$ at 4°C for 5 min and the supernatant was used for the measurement of O_2^- production. O_2^- production was calculated using a millimolar extinction coefficient of 19.1.

2.3. Measurement of intracellular free Ca^{2+} concentrations with fura-2

Differentiated HL-60 cells were washed three times with Hanks' solution. The cells ($1-5 \times 10^6$/ml) were treated with 1 μM fura-2/AM at 37°C for 15 min and were then centrifuged in order to remove the remaining fura-2/AM and washed twice with Hanks' solution. The cells were suspended in Hanks' solution at concentrations of $1-5 \times 10^6$/ml, and 1.5 ml of the cell suspension was used for the fura-2 assay. Fluorescence of fura-2 at 510 nm by excitation waves at 340 and 380 nm was monitored simultaneously by a fluorospectrophotometer (Hitachi, F-2000), described previously (Nakahata et al., 1994). The maximum ratio of fluorescence was obtained in the presence of 0.1% Triton X-100 and the minimum ratio of fluorescence was obtained in the presence of 3 mM EGTA. Free calcium concentrations were calculated using the K_d (224 nM) of fura-2 to Ca^{2+}.

2.4. Assay of [^{3}H]inositol phosphates

Phosphoinositide hydrolysis was measured by determination of [^{3}H]inositol phosphates, described previously (Nakahata et al., 1989). In brief, 48 h after differentiation, *myo*-[2-^{3}H(*N*)]inositol was added to the differentiating medium to make a final concentration of 2 μCi/ml and cultured for an additional 24 h. The cells were washed three times with Hanks' solution. The reactions were carried out at 37°C in glass tubes containing $1-5 \times 10^6$ cells in Hanks' solution for 15 min in the presence of 10 mM LiCl. The reactions were terminated by the addition of trichloroacetic acid to make a final concentration of 5%. The trichloroacetic acid extract was washed three times with ether and applied to an anion exchange column (AG 1X-8, formate form). Total [^{3}H]inositol phosphates were eluted by 1 M ammonium formate in 0.1 M formic acid and counted by liquid scintillation counting.

2.5. Measurement of phospholipase A_2 activity

Phospholipase A_2 activity was determined by measuring [^{3}H]arachidonic acid released from HL-60 cells (Xing and Mattera, 1992). In brief, at the end of differentiation, HL-60 cells suspended in Hanks' solution containing 3 mg/ml bovine serum albumin were labeled with

[^{3}H]arachidonic acid (1 μCi/ml) for 1 h. The cells were washed three times with Hanks' solution. The reactions were carried out at 37°C in glass tubes containing 1–5 × 10^6 cells in 0.4 ml Hanks' solution for 15 min in the presence of 0.2 mM cold arachidonic acid and 3 mg/ml bovine serum albumin. The reactions were terminated by addition of 3.6 ml ice-cold solution (composition, Tris-HCl 50 mM, pH 7.5, KCl 100 mM, EGTA 5 mM, EDTA 5 mM). The samples were centrifuged at 1400 × g at 4°C for 5 min and 2 ml of the supernatant was counted by liquid scintillation counting.

2.6. Measurement of phospholipase D activity

Phospholipase D activity was determined by measuring [^{3}H]phosphatidylethanol. In brief, 48 h after differentiation, [^{3}H]palmitic acid was added to the differentiating medium to make a final concentration of 5 μCi/ml and cultured for an additional 24 h. The cells were washed three times with Hanks' solution. The reactions were carried out at 37°C in glass tubes containing 1–2 × 10^6 cells in Hanks' solution for 15 min in the presence of ethanol (0.5%, v/v). The reactions were terminated by additions of 1.5 ml chloroform/methanol (1 : 2). Cellular lipids were extracted by the method of Bligh and Dyer (1959). The lower chloroform phase was dried and spotted on LK5D silica gel plates (Whatman). The samples were developed by using the upper phase of a solvent system consisting of ethyl acetate/isooctane/acetic acid/water (110 : 50 : 20 : 100, by volume). The authentic phosphatidylethanol was used as standard and visualized with iodine vapor. Spots corresponding to phosphatidylethanol were scraped off and counted by liquid scintillation counting.

2.7. Materials

N-Formyl-Met-Leu-Phe (fMLP), phorbol 12-myristate 13-acetate (PMA), N^6,2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate (dibutyryl cAMP), superoxide dismutase, arachidonic acid, phosphatidylcholine and *N*-ethylmaleimide were purchased from Sigma (St. Louis, MO, USA). 2,5-Tert-butyl-1,4-benzohydroquinone (tBuBHQ), transferrin, insulin, sodium selenite, cytochrome *c* from horse heart and Triton X-100 were obtained from Wako Pure Chemicals (Osaka, Japan). RPMI 1640 was from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum was from Bioserum (Victoria, Australia). Fura-2/AM, *O*,*O*′-bis(2-aminophenyl)ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetraacetoxymethyl ester (BAPTA/AM), EGTA and Hepes were from Dojindo (Kumamoto, Japan). Phosphatidylethanol was from Funakoshi (Tokyo, Japan). *myo*-[2-^{3}H(*N*)]Inositol, [^{3}H]arachidonic acid and [^{3}H]palmitic acid was from NEN/DuPont (Boston, MA, USA). Other chemicals and drugs were of reagent grade or the highest quality available.

3. Results

fMLP and PMA are known to induce O_2^- production. The time course of O_2^- production elicited by tBuBHQ, a Ca^{2+} pump inhibitor of the intracellular Ca^{2+} store, was compared with that of fMLP or PMA (Fig. 1). fMLP (0.1 μM) induced O_2^- production transiently, showing a typical receptor-mediated activation. PMA (0.1 μM)-induced O_2^- production was linear from 2 min to over 15 min after its treatment. PMA had a lag time of about 2 min before producing O_2^-. On the other hand, tBuBHQ induced O_2^- production rapidly within 30 s.

As shown in Fig. 2, the effects of fMLP, PMA and tBuBHQ on $[Ca^{2+}]_i$ were examined by a fura-2 assay. fMLP mobilized Ca^{2+} transiently with a peak of 5 s. However, no change in $[Ca^{2+}]_i$ was detected by PMA at all, suggesting the presence of a pathway to NADPH oxidase activation without increasing $[Ca^{2+}]_i$. tBuBHQ caused a slow Ca^{2+} mobilization, i.e. the time course of the Ca^{2+} mobilization was different from that of the O_2^- production (Fig. 1).

The concentration dependencies of O_2^- production, Ca^{2+} mobilization and phosphoinositide hydrolysis by these stimuli are shown in Fig. 3. fMLP caused O_2^- production, Ca^{2+} mobilization and phosphoinositide hydrolysis in a concentration-dependent manner with EC_{50} values of 10.1 nM, 0.68 nM and 3.1 nM, respectively. Therefore, Ca^{2+} mobilization was more sensitive than O_2^- production in response to fMLP. The result might suggest that an increase in $[Ca^{2+}]_i$ alone is not enough to produce O_2^-, and the pathways other than an increase in $[Ca^{2+}]_i$ are necessary for fMLP-induced O_2^- production. While PMA activated O_2^- production in a concentration-dependent

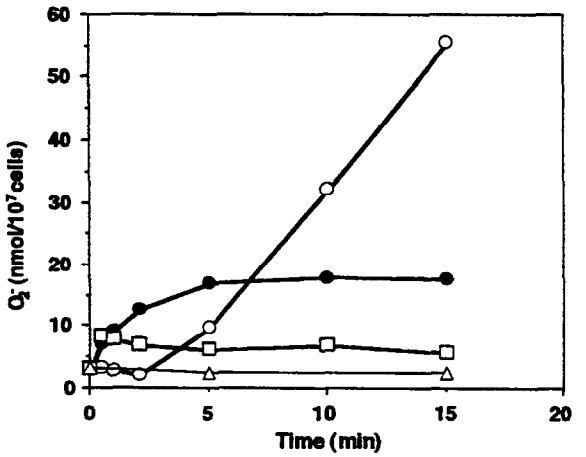

Fig. 1. Time courses of O_2^- production by fMLP, PMA and tBuBHQ in neutrophil-like differentiated HL-60 cells. The cells suspended in Hanks' solution were incubated at 37°C with 0.1 μM fMLP (●), 0.1 μM PMA (○), 1 μM tBuBHQ (□) or vehicle (△) for the indicated time. O_2^- production was determined by measuring absorbance of cytochrome *c* at 549 nm and 540 nm. Each point represents the mean of two determinations.

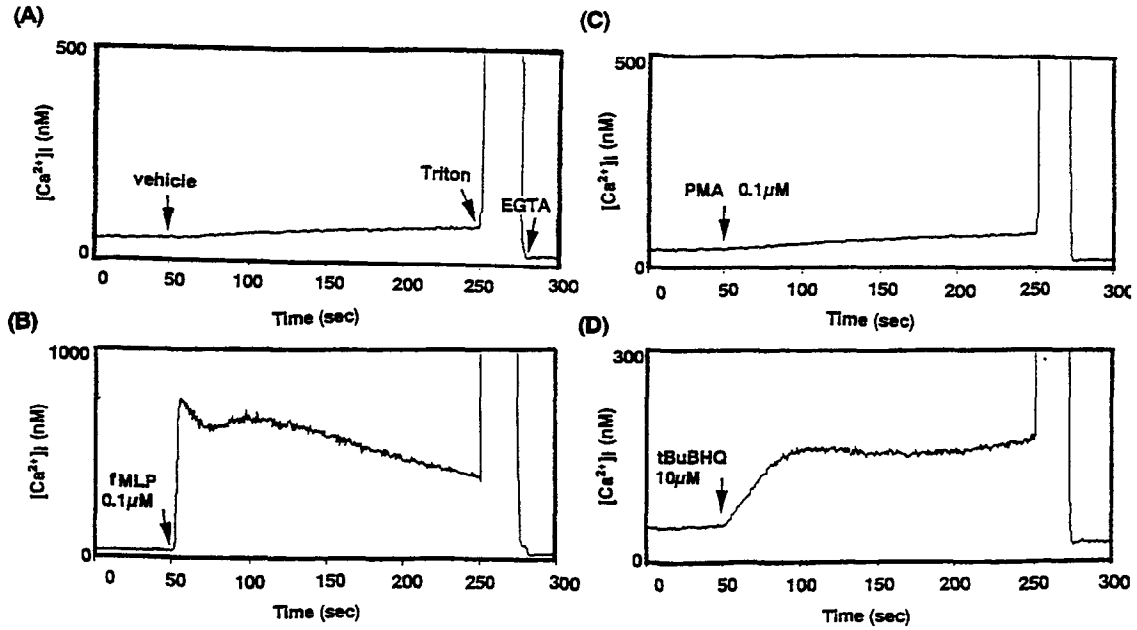

Fig. 2. Effects of fMLP, PMA and tBuBHQ on intracellular free Ca^{2+} concentration. HL-60 cells loaded with fura-2 were suspended in Hanks' solution, and drugs were added to the cell suspensions at 50 s. (A) vehicle; (B) 0.1 μM fMLP; (C) 0.1 μM PMA; (D) 10 μM tBuBHQ. The maximum and minimum ratios of fluorescence of fura-2 were obtained by addition of Triton X-100 (0.1%) and EGTA (3 mM) at 250 and 275 s.

manner, it did not cause phosphoinositide hydrolysis and Ca^{2+} mobilization. The concentration-response curves for tBuBHQ in O_2^- generation and Ca^{2+} mobilization were very similar, although tBuBHQ did not accumulate inositol phosphates.

The effect of extracellular Ca^{2+} on fMLP- or tBuBHQ-induced Ca^{2+} mobilization was examined using EGTA (Fig. 4). In the presence of EGTA, fMLP still increased $[Ca^{2+}]_i$ but did not sustain the increased level, suggesting that fMLP causes Ca^{2+} release from the intracellular Ca^{2+} store followed by an influx from extracellular medium in the presence of extracellular Ca^{2+}. On the other hand, tBuBHQ-induced increase in $[Ca^{2+}]_i$ was also attenuated in the presence of EGTA, suggesting that tBuBHQ influxed Ca^{2+} together with the inhibition of Ca^{2+} uptake to intracellular Ca^{2+} storage sites by an inhibition of the Ca^{2+} pump. In the same condition, i.e. extracellular Ca^{2+} ions were chelated by 3 mM EGTA, O_2^- production induced by fMLP was reduced significantly (Fig. 5), suggesting that fMLP-induced O_2^- production might be partly dependent on extracellular Ca^{2+}. However, neither PMA- nor tBuBHQ-induced O_2^- production was suppressed by EGTA treatment.

After the cells were loaded with 10 μM BAPTA/AM at 37°C for 30 min, intracellular Ca^{2+} was chelated by BAPTA, i.e. the $[Ca^{2+}]_i$ level of the cells was kept lower

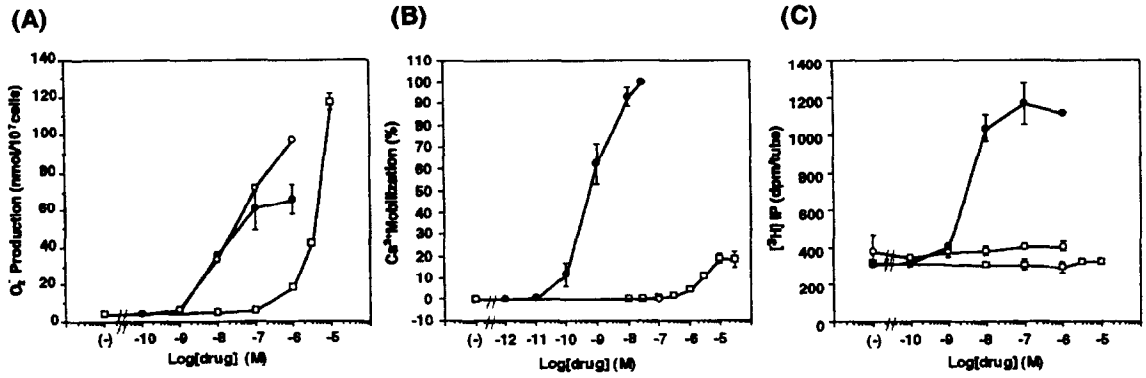

Fig. 3. Concentration-response curves for fMLP, PMA and tBuBHQ in O_2^- production, Ca^{2+} mobilization and phosphoinositide hydrolysis. Concentration-response curves for fMLP (●), PMA (○) and tBuBHQ (□) are shown in each panel. (A) O_2^- production of the cells treated with drugs for 15 min at 37°C. Results represent the mean ± S.E. from six determinations. (B) Ca^{2+} mobilization. Results are expressed as the percentage of fMLP-induced maximal response and represent the mean ± S.E. of three determinations. (C) Phosphoinositide hydrolysis of the cells treated with drugs for 15 min. [^{3}H]Inositol phosphates (IP) were measured as described in Materials and methods. Results represent the mean ± S.E. of six determinations.

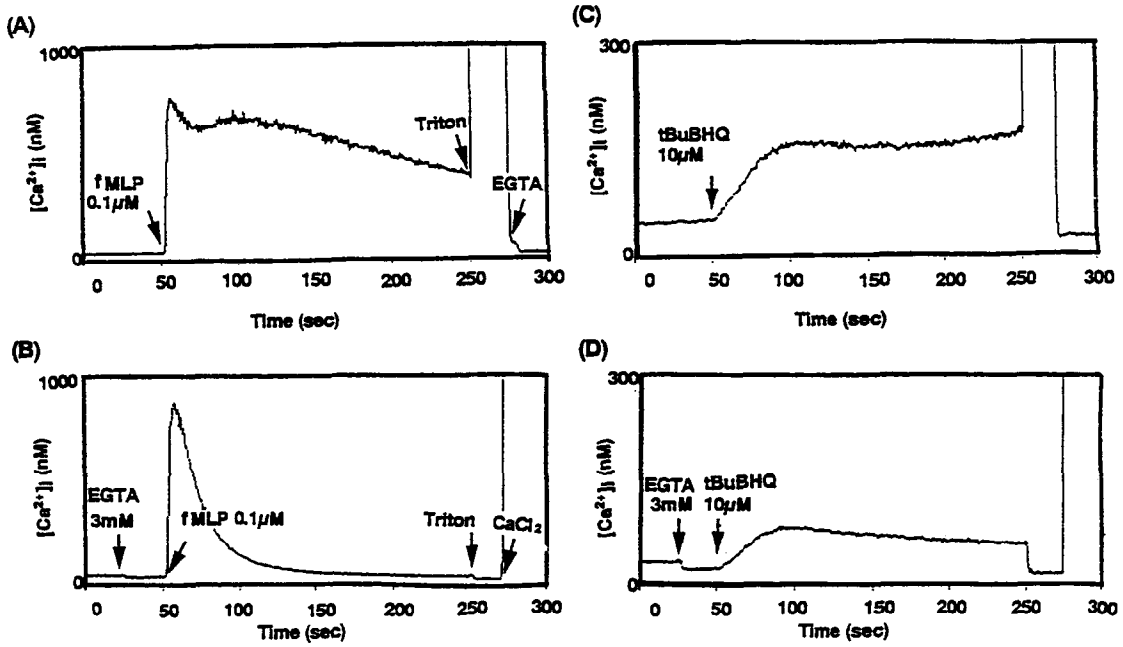

Fig. 4. Effect of EGTA on fMLP- and tBuBHQ-induced Ca^{2+} mobilization. In the presence of extracellular Ca^{2+} (1.2 mM), 0.1 μM fMLP (A) or 10 μM tBuBHQ (C) induced Ca^{2+} mobilization. In the presence of EGTA (3 mM), 0.1 μM fMLP (B) or 10 μM tBuBHQ (D) induced Ca^{2+} mobilization in a different manner from that in the presence of Ca^{2+}.

than that of non-treated cells (Fig. 6). fMLP- and tBuBHQ-induced Ca^{2+} mobilizations were inhibited completely by BAPTA/AM treatment. In the cells treated with BAPTA/AM, fMLP did not induce O_2^- production at all. Therefore, fMLP-induced O_2^- production is solely dependent on intracellular Ca^{2+}. Interestingly, PMA-induced O_2^- production was about 50% inhibited by BAPTA/AM treatment. Since PMA did not increase $[Ca^{2+}]_i$, O_2^- production induced by PMA might be partly dependent on a resting level of $[Ca^{2+}]_i$ in normal cells. On the other hand, tBuBHQ-induced O_2^- production was not suppressed at all by BAPTA/AM treatment. According to the results of EGTA and BAPTA/AM treatments, tBuBHQ-induced O_2^- production is independent of an increase in $[Ca^{2+}]_i$.

To examine the action of the Ca^{2+} pump inhibitor, thapsigargin, another Ca^{2+} pump inhibitor, was used (Fig. 7). While thapsigargin increased $[Ca^{2+}]_i$ more potently than tBuBHQ, it scarcely induced O_2^- production. The results support the idea that (1) an increase in $[Ca^{2+}]_i$ alone is not enough to cause O_2^- production, and (2) tBuBHQ induces O_2^- production through a Ca^{2+}-independent process.

To clarify the site of action of Ca^{2+}, the Ca^{2+} dependency of fMLP-induced activation of phospholipase A_2 or phospholipase D was examined by using BAPTA/AM. As shown in Fig. 8, fMLP liberated arachidonic acid in a concentration-dependent manner. In the cells treated with BAPTA/AM, fMLP (0.1 μM) did not liberate arachidonic acid at all, suggesting that fMLP-induced phospholipase A_2 activation depends completely on intracellular Ca^{2+}. PMA (0.1 μM) and tBuBHQ (10 μM), however, did not liberate arachidonic acid. In addition, phospholipase D activity was determined by measuring [^{3}H]phosphatidylethanol, a metabolite of phosphatidylcholine by phospholipase D in the presence of ethanol (Fig. 9). fMLP induced phosphatidylethanol accumulation in a concentration-dependent manner. BAPTA/AM treatment completely inhibited fMLP-induced phosphatidylethanol accumulation, suggesting that fMLP-induced phospholipase D activation also depends on intracellular Ca^{2+}. PMA (0.1 μM) also induced phosphatidylethanol accumulation, which was partly suppressed by BAPTA/AM treatment.

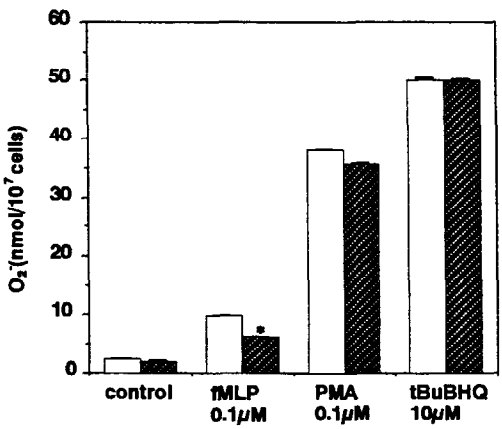

Fig. 5. Effect of EGTA on O_2^- production induced by fMLP, PMA or tBuBHQ. The cells suspended in Hanks' solution were incubated for 15 min with drugs in the presence (hatched column) or absence (open column) of 3 mM EGTA. Results represent the mean ± S.E. of three determinations. * Significant difference between the values in the presence and absence of EGTA ($P < 0.05$).

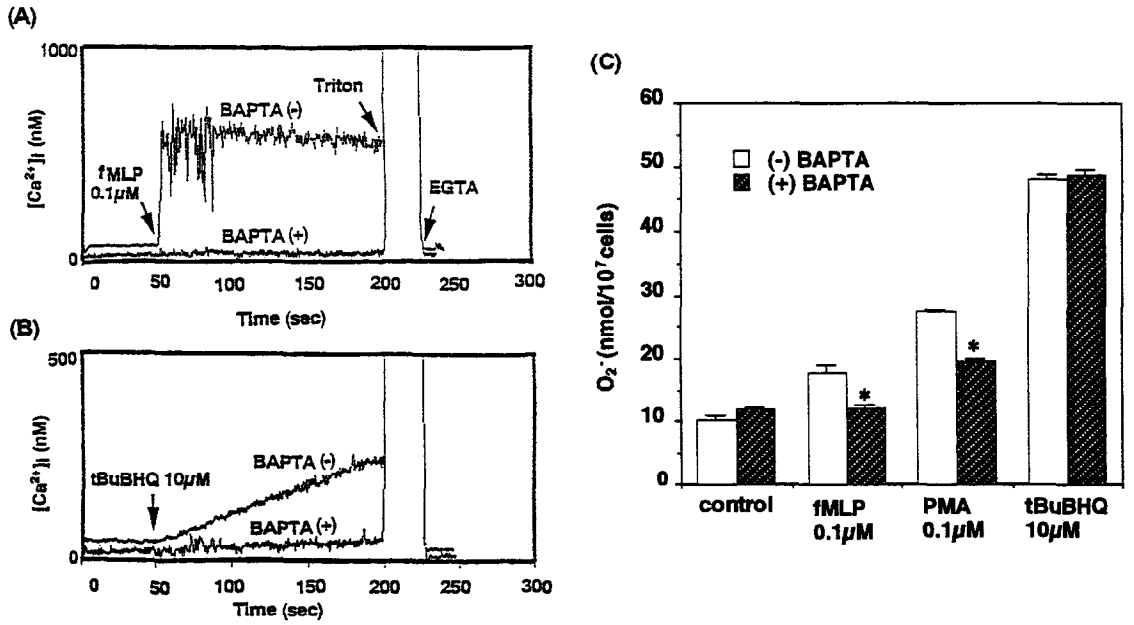

Fig. 6. Effect of BAPTA on Ca^{2+} mobilization or O_2^- production. The cells suspended in Hanks' solution were loaded with 10 μM BAPTA/AM at 37°C for 30 min. (A) Ca^{2+} mobilization induced by 0.1 μM fMLP with or without BAPTA/AM treatment. (B) Ca^{2+} mobilization induced by 10 μM tBuBHQ with or without BAPTA/AM treatment. (C) O_2^- production induced by 0.1 μM fMLP, 0.1 μM PMA or 10 μM tBuBHQ with (hatched column) or without (open column) 10 μM BAPTA/AM treatment. Results represent the mean ± S.E. of three determinations. * Significant difference between the values with and without BAPTA/AM treatment ($P < 0.05$).

However, tBuBHQ (10 μM) did not accumulate phosphatidylethanol.

To elucidate the mechanism of tBuBHQ-induced O_2^- production, phosphatidylcholine was incubated with fMLP, PMA or tBuBHQ in the absence of the cells (Fig. 10). tBuBHQ, but not fMLP or PMA, effectively caused O_2^- production in this cell-free system.

4. Discussion

We tried to clarify the contribution of intracellular Ca^{2+} to O_2^- production by using intracellular Ca^{2+} chelator, BAPTA. The comparative study of fMLP, PMA and tBuBHQ on Ca^{2+} mobilization and O_2^- production in neutrophil-like differentiated HL-60 cells indicates that (i) intracellular Ca^{2+} mobilization is essential for fMLP-induced O_2^- production, (ii) intracellular Ca^{2+} mobilization is not absolutely necessary for PMA-induced O_2^- production, and (iii) intracellular Ca^{2+} mobilization occurs independently of tBuBHQ-induced O_2^- production.

tBuBHQ mobilized Ca^{2+} and induced O_2^- production. Since (i) the time course of O_2^- production of tBuBHQ is quite different from that of Ca^{2+} mobilization, and (ii) EGTA and BAPTA/AM treatment does not affect O_2^- production induced by tBuBHQ, O_2^- production induced by tBuBHQ is not due to Ca^{2+} mobilization. The fact that thapsigargin, another Ca^{2+} pump inhibitor which mobi-

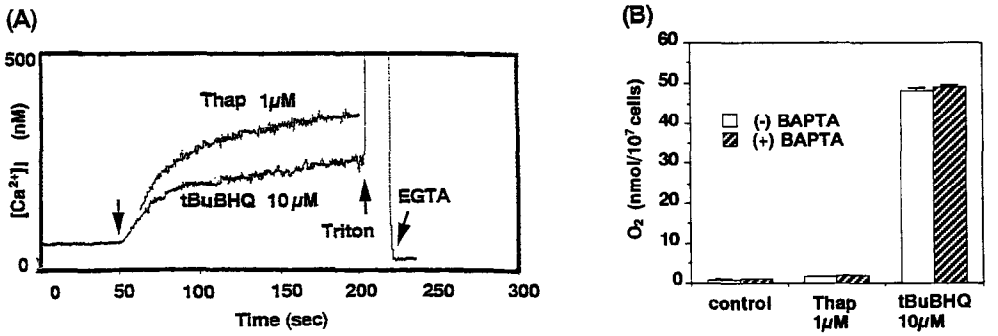

Fig. 7. Comparative study of thapsigargin with tBuBHQ in Ca^{2+} mobilization and O_2^- production. (A) Ca^{2+} mobilization induced by thapsigargin and tBuBHQ. (B) O_2^- production induced by thapsigargin and tBuBHQ for 15 min incubation. Open column: control; hatched column: 10 μM BAPTA/AM treatment. Results represent the mean ± S.E. of three determinations.

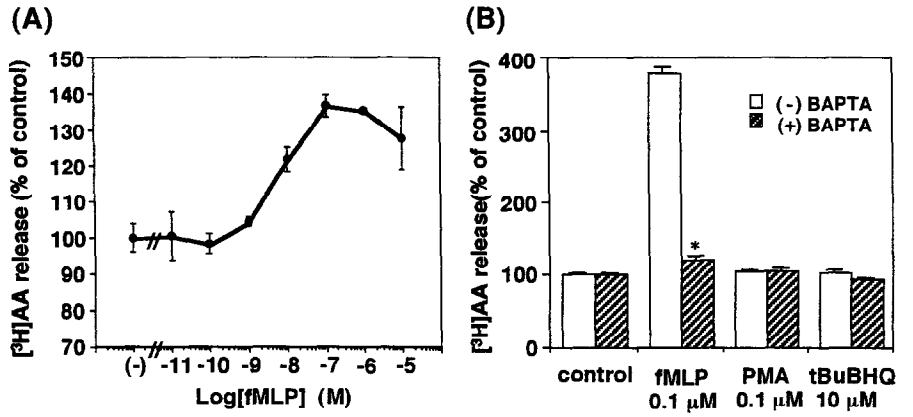

Fig. 8. Effect of BAPTA on phospholipase A_2 activity. The cells suspended in Hanks' solution were loaded with 10 μM BAPTA/AM at 37°C for 30 min. [^{3}H]Arachidonic acid (AA) release from the cells for 15 min was measured as described in Materials and methods. (A) Concentration-response curve for fMLP in arachidonic acid release. (B) Effect of BAPTA on arachidonic acid release induced by fMLP, PMA or tBuBHQ. [^{3}H]Arachidonic acid release was measured in normal (open column) or BAPTA/AM treated (hatched column) cells. Results represent the mean ± S.E. of three determinations. * Significant difference between the values with and without BAPTA/AM treatment ($P < 0.05$).

lizes Ca^{2+} much more than tBuBHQ, does not induce O_2^- production in cyclic AMP-differentiated HL-60 cells, indicates that an increase in $[Ca^{2+}]_i$ by tBuBHQ does not contribute to O_2^- production. tBuBHQ caused O_2^- production when it was incubated with phosphatidylcholine in a cell-free system. Therefore, tBuBHQ interacts with phospholipids forming O_2^- chemically, like other quinone derivatives (Powis et al., 1981).

fMLP is known to activate phosphoinositide-specific phospholipase C, resulting in an accumulation of IP_3 followed by intracellular Ca^{2+} mobilization (Kikuchi et al., 1986). Since fMLP caused phosphoinositide hydrolysis and Ca^{2+} mobilization with a similar concentration dependency (Fig. 3), fMLP-induced Ca^{2+} mobilization could be connected to phosphoinositide hydrolysis. Furthermore, the Ca^{2+}-dependent process is an essential step in fMLP-induced O_2^- production, because EGTA and BAPTA/AM reduce fMLP-induced O_2^- production. The results that BAPTA/AM treatment completely inhibited fMLP-induced phospholipase A_2 (Fig. 8) and phospholipase D activation (Fig. 9) as well as O_2^- production, indicate that activation of these enzymes might lies downstream of Ca^{2+} mobilization. These results are consistent with the observations that fMLP activates phospholipase A_2 in a Ca^{2+}-dependent fashion to liberate arachidonic acid (Okajima and Ui, 1984; Xing and Mattera, 1992; Xing et al., 1994), and that fMLP also activates phospholipase D Ca^{2+}-dependently to produce phosphatidic acid (Gelas et al., 1992). Since the concentration-response curve of Ca^{2+} mobilization for fMLP is left to the curves of O_2^- production, phospholipase A_2 activation and phospholipase D activation, an increase in $[Ca^{2+}]_i$ alone may be not enough to activate phospholipase A_2 and phospholipase D and to induce O_2^- production in response to fMLP. In fact, the

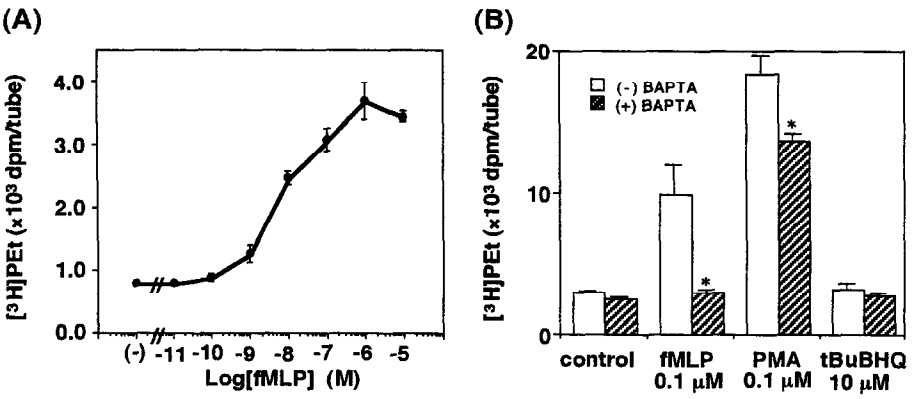

Fig. 9. Effect of BAPTA on phospholipase D activity. [^{3}H]Phosphatidylethanol (PEt) accumulation of the cells in the presence of ethanol (0.5%, v/v) for 15 min was measured as described in Materials and methods. (A) Concentration-response curve for fMLP in phosphatidylethanol accumulation. (B) Effect of BAPTA on phosphatidylethanol accumulation induced by fMLP, PMA or tBuBHQ. [^{3}H]Phosphatidylethanol accumulation was measured in normal (open column) or BAPTA/AM treated (hatched column) cells. Results represent the mean ± S.E. of three determinations. * Significant difference between the values with and without BAPTA/AM treatment ($P < 0.05$).

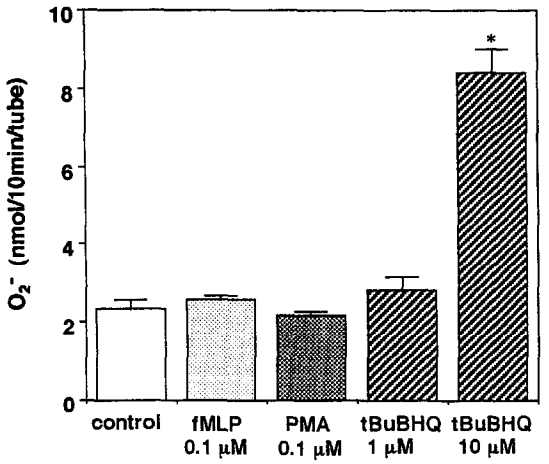

Fig. 10. Direct interaction of tBuBHQ with phospholipids to induce O_2^- production. Phosphatidylcholine (25 μg) was incubated with fMLP (0.1 μM), PMA (0.1 μM) or tBuBHQ (1 and 10 μM) in the absence of cells at 37°C for 10 min. Results represent the mean ± S.E. of three determinations. * Significant difference from control ($P < 0.05$).

activation of phospholipase A_2 and phospholipase D by fMLP is mediated through receptor-stimulated pertussis toxin-sensitive G-protein (Billah et al., 1989; Xing and Mattera, 1992). The activation of phospholipase A_2 and phospholipase D by fMLP occurs in parallel to phosphoinositide-specific phospholipase C activation. Thus, Ca^{2+} supplied from the phosphoinositide-specific phospholipase C-IP_3 pathway in response to fMLP could be utilized as a cofactor for activation of phospholipase A_2 and phospholipase D upstream of O_2^- production.

PMA, a protein kinase C activator, caused O_2^- production without changing $[Ca^{2+}]_i$. Recent studies show that protein kinase C phosphorylates $p47^{phox}$, which is necessary for NADPH oxidase activation (Curnutte et al., 1994). In BAPTA/AM- but not EGTA-treated cells, PMA-induced O_2^- production was inhibited partially but significantly. Therefore, PMA-induced O_2^- production may be caused by activation of both Ca^{2+}-dependent and -independent protein kinase C isoforms. Although the β-type of protein kinase C is involved in O_2^- production (Curnutte et al., 1994), it remains unknown whether other isoforms of protein kinase C contribute to O_2^- production. The mechanism of O_2^- production involving Ca^{2+} utilization induced by PMA was clearly different from that induced by fMLP. Pharmacological studies using phosphorylation inhibitors reveal that fMLP-induced O_2^- production is mediated through genistein-sensitive kinase, and the PMA-induced one is mediated through staurosporine-sensitive kinase (Chanock et al., 1994). In the present study, it is shown that PMA activates phospholipase D but not phospholipase A_2. It is possible that phospholipase A_2 activation is not absolutely required for O_2^- production induced by PMA. Phospholipase D activation induced by PMA may constitute a positive feedback loop; activation of phospholipase D by protein kinase C produces phosphatidic acid, which is converted to 1,2-diacylglycerol by phosphatidic acid phosphohydrolase (Suchard et al., 1994), and 1,2-diacylglycerol can stimulate protein kinase C again. Thus, O_2^- production by PMA is thought to be positively regulated by the phospholipase D-protein kinase C pathway. Our present study demonstrates that both O_2^- production and phospholipase D are activated by both Ca^{2+}-dependent and -independent protein kinase C isoforms.

In conclusion, tBuBHQ causes O_2^- production by direct interaction with phospholipids, although it increases $[Ca^{2+}]_i$ by inhibition of the Ca pump. Thus we should use tBuBHQ carefully when applying it in the experimental system associated with O_2^- production. fMLP induces O_2^- production through a Ca^{2+}-dependent process, and Ca^{2+} supplied from the phosphoinositide hydrolysis pathway could be at least utilized as a cofactor for activation of phospholipase A_2 and phospholipase D upstream of protein kinase C activation and O_2^- production.

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