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Effects of Hydroquinone-Type and Phenolic Antioxidants on Calcium Signals and Degranulation of RBL-2H3 Cells

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ABSTRACT. We previously reported that a hydroquinone-type antioxidant, 2,5-di(*tert*-butyl)-1,4-hydroquinone (DTBHQ), increases intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), causes degranulation together with a protein kinase C activator, phorbol 12-myristate 13-acetate (TPA), and increases antigen-induced degranulation in rat basophilic leukemia (RBL-2H3) cells. In this study, the effects of five hydroquinone-type and phenolic antioxidants (2,5-di(*tert*-amyl)-1,4-hydroquinone [DTAHQ], 2-*tert*-butyl-1,4-hydroquinone [MTBHQ], 3,5-di(*tert*-butyl)-4-hydroxytoluene [BHT], 3,5-di(*tert*-butyl)-4-hydroxyanisole [DTBHA], and 3-*tert*-butyl-4-hydroxyanisole [MTBHA]) on $[Ca^{2+}]_i$ and degranulation (β-hexosaminidase release) were examined and compared with that of DTBHQ. DTAHQ (≥3 μM) showed effects similar to those of DTBHQ (10 μM) on $[Ca^{2+}]_i$ elevation, induction of degranulation with TPA, and increase of antigen-induced degranulation. BHT (50 μM) and DTBHA (50 μM) caused $[Ca^{2+}]_i$ elevation and increased degranulation in the presence of TPA or antigen, but their effects were less than those of DTBHQ and DTAHQ. MTBHQ and MTBHA had no effect on $[Ca^{2+}]_i$ and degranulation, even at 50 μM. The degree of Ca^{2+} response caused by the compounds correlated well with the increase in degranulation, but not with their antioxidant activity estimated with the first oxidation potential. From these results, it is suggested that the increasing effects of six antioxidants on degranulation in the presence of TPA or antigen were dependent on $[Ca^{2+}]_i$ increase caused by the compounds, probably through their ability to inhibit endoplasmic reticulum Ca^{2+}-ATPase. BIOCHEM PHARMACOL 51;11:1513–1519, 1996.

KEY WORDS. basophilic leukemia cell; cytosolic calcium level; degranulation; hydroquinone-type antioxidant; phenolic antioxidant

In the immediate hypersensitivity reaction, the high-affinity IgE receptors (FcεRI) on the surfaces of mast cells and basophils mediate degranulation of the cells and release of inflammatory mediators, such as histamine [1]. This FcεRI-mediated degranulation is entirely dependent on extracellular Ca^{2+} [2] and requires elevation of cytosolic Ca^{2+} levels [3]. It has been suggested that antigen stimulates Ca^{2+} influx across the plasma membranes, as well as Ca^{2+} release from intracellular Ca^{2+} storage sites [3].

Three unrelated compounds, thapsigargin [4], cyclopiazonic acid [5], and a widely used antioxidant, DTBHQ§ [6], are known to elevate cytosolic Ca^{2+} levels by preventing Ca^{2+} reuptake into the endoplasmic reticulum pool and increasing the Ca^{2+} influx from the extracellular medium through activation of Ca^{2+} release-activated channel. Their mechanism of action appears to involve selective inhibition of phosphoenzyme intermediate formation on the endoplasmic reticulum Ca^{2+}-ATPase [7]. These three compounds have been reported to induce interleukin-2 synthesis and cell proliferation in human T cells [8] and to stimulate DNA synthesis in Swiss 3T3 cells synergistically with a phorbol ester [9].

Thapsigargin has been shown to induce the Ca^{2+} response in rat peritoneal mast cells [10, 11] and in RBL-2H3 cells [12, 13]. Recently, we have shown that DTBHQ causes elevation of the cytosolic Ca^{2+} level, increases antigen-induced degranulation (histamine secretion), and induces degranulation of RBL-2H3 cells in the presence of a protein kinase C activator, phorbol 12-myristate 13-acetate (TPA) [14].

Other phenolic antioxidants have also been reported to show biological activities. BHT and DTBHA inhibit (Ca^{2+}, Mg^{2+})-ATPase of the sarcoplasmic reticulum [15]. MTBHA inhibits tumor necrosis factor-induced cytotoxicity [16]. In this study, we investigated the effects of five antioxidants that have structures similar to that of DTBHQ,

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§ *Abbreviations:* DTBHQ, 2,5-di(*tert*-butyl)-1,4-hydroquinone; DTAHQ, 2,5-di(*tert*-amyl)-1,4-hydroquinone; BHT, 3,5-di(*tert*-butyl)-4-hydroxytoluene; DTBHA, 3,5-di(*tert*-butyl)-4-hydroxyanisole; MTBHQ, 2-*tert*-butyl-1,4-hydroquinone; MTBHA, 3-*tert*-butyl-4-hydroxyanisole; TPA, phorbol 12-myristate 13-acetate; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration.

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DTAHQ, MTBHQ, BHT, DTBHA, and MTBHA (Fig. 1), on the intracellular Ca^{2+} level and degranulation of RBL-2H3 cells in the presence of antigen or TPA, and compared these effects with those of DTBHQ.

MATERIALS AND METHODS

Reagents, Buffer, and Cells

DTBHQ and DTAHQ were kindly supplied by Ouchi Shinko Chemical Industries Co. (Tokyo, Japan). BHT and MTBHA were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DTBHA and MTBHQ were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). Fura-2 AM was purchased from Dojindo Laboratories (Kumamoto, Japan). Ionomycin and phorbol 12-myristate 13-acetate (TPA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Murine anti-dinitrophenyl (DNP) monoclonal IgE antibody (IgE-53-569) and dinitrophenylated BSA (DNP_7-BSA) were prepared as described previously [17]. All other reagents were of the best grade commercially available. The PIPES buffer used in this study consisted of 140 mM NaCl, 5 mM KCl, 0.6 mM $MgCl_2$, 1 mM $CaCl_2$, 5.5 mM glucose, 0.1% (w/v) BSA, and 10 mM PIPES (pH 7.4). RBL-2H3 cells [18], a secreting subline of rat basophilic leukemia cells, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum.

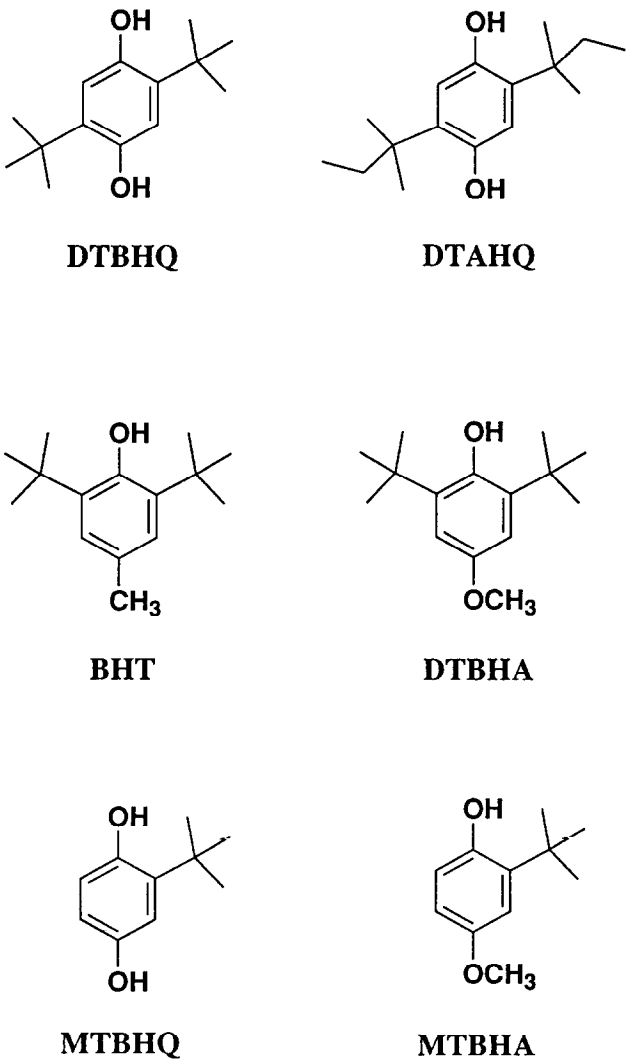


FIG. 1. Structures of the six antioxidants used in this paper.

Measurement of Intracellular Free Ca^{2+} Concentration ($[Ca^{2+}]_i$)

RBL-2H3 cells (6×10^5 cells/mL) were loaded with Fura-2 AM (6 μM) as described previously [19]. After removal of free dye by centrifugation, the cells were resuspended in 1.5 mL of the PIPES buffer and illuminated alternately at two excitation wavelengths (335 and 362 nm). Fluorescence at 495 nm was measured in a 1-cm quartz cuvette with a Shimadzu RF-5000 spectrofluorophotometer with stirring at 37°C, and the ratio of the fluorescence strength obtained at these two excitation wavelengths was calculated and used as an indiation of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was estimated by the method described by Grynkiewicz *et al.* [20]. In the case of antigen stimulation, the cells were loaded simultaneously with anti-DNP IgE and Fura-2 AM.

Measurement of β-Hexosaminidase Activity

The activity of β-hexosaminidase released from the cells was measured as follows. RBL-2H3 cells were preincubated for 24 hr at 37°C in a 24-well flat-bottom microtiter plate in 1 mL of DMEM containing 10% (v/v) fetal calf serum. The supernatants were discarded, and the cells (2×10^5 cells/well) were washed 3 times with PIPES buffer. The cells were then incubated in 500 μL of the buffer containing each drug for 30 min at 37°C. The supernatant was withdrawn from each well. To 50 μL of supernatant, 200 μL of substrate solution [1.3 mg of *p*-nitrophenyl-2-acetamide-2-deoxy-β-glucopyranoside per mL of 0.1 M sodium citrate buffer (pH 4.5)] was added, and the mixture was incubated for 60 min at 37°C. The reaction was terminated by addition of 500 μL of 0.2 M glycine (adjusted to pH 10.0 with 1 N NaOH), and absorbance at 405 nm was measured. To quantify the enzyme activity remaining in the cells, they were then treated with 500 μL of 0.2% (v/v) Triton X-100, and the extract was analyzed as described above. In the case of antigen stimulation, the cells were incubated with anti-DNP IgE for 1 hr at 37°C and washed 3 times with PIPES buffer. Then, 400 μL of drug solution was added to the cells and they were incubated for 10 min at 37°C. Finally, 100 μL of DNP_7-BSA (50 μg/mL) was added, and the cells were incubated for 40 min at 37°C.

Measurement of First Oxidation Potentials

First oxidation potentials of six antioxidants were measured with BAS100B Electrochemical Analyzer (Bioanalytical System). Acetonitrile containing 0.1 M tetraethyl-

ammonium perchlorate was used as solvent. After transferring the solution containing the test chemical into a cell, oxygen was purged by bubbling N_2 gas for 10 min. Cyclic voltamograms were recorded at a scan rate of 100 mV/s; the test solution was maintained under a steady stream of N_2 gas. The reference electrode used was Ag/Ag^+ electrode.

RESULTS

Effects of the Compounds on $[Ca^{2+}]_i$

DTBHQ induced a rapid and sustained increase in $[Ca^{2+}]_i$ at a concentration of 10 μM in RBL-2H3 cells as reported previously [14] (Fig. 2a). When the fluorescence ratios in resting cells and cells treated with ionomycin (0.5 μM) were taken as 0 and 100%, respectively, DTBHQ (10 μM) increased $[Ca^{2+}]_i$ by *ca.* 86%. DTAHQ also induced a Ca^{2+} response at 10 μM as did DTBHQ (Fig. 2b). On the other hand, BHT and DTBHA induced no or little increase in $[Ca^{2+}]_i$ at 10 μM. However, at 50 μM, they induced weak Ca^{2+} responses (Fig. 2c, d). As shown in Fig. 2e and f, MTBHQ and MTBHA did not induce any Ca^{2+} response, even at 50 μM (the F_{335}/F_{362} ratio rose slowly without stimulation).

Figure 3 shows representative dose-response curves of the fluorescence ratio 100 sec after the addition of each compound. Ca^{2+} responses induced by DTAHQ, BHT, and DTBHA were dose-dependent, as was that induced by DTBHQ. The effects of DTAHQ and DTBHQ were stronger than those of BHT and DTBHA. At doses of less than 10 μM, DTAHQ showed a stronger effect than DTBHQ. The concentrations of DTBHQ and DTAHQ that gave half-maximal increases (ED_{50}) were 2.9 μM and 0.8 μM, respectively, when the basal and maximal $[Ca^{2+}]_i$ were taken as 62 nM and 220 nM, respectively.

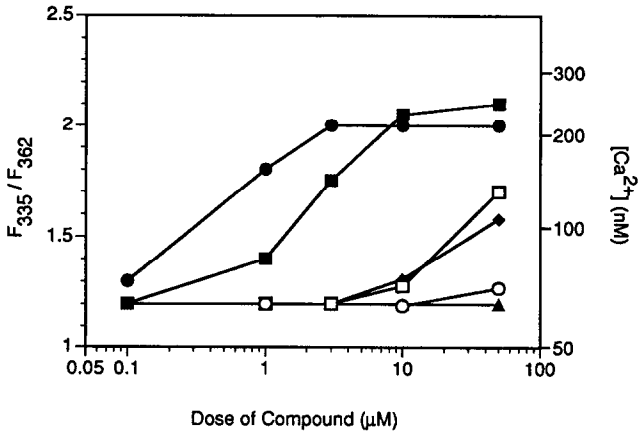


FIG. 3. Dose-dependency of the increase in $[Ca^{2+}]_i$ of RBL-2H3 cells induced by DTBHQ (■), DTAHQ (●), MTBHQ (▲), BHT (♦), DTBHA (□), and MTBHA (○). Values at 100 sec after the addition of the drugs are plotted against the drug concentration. In every experiment, the fluorescence ratio (F_{335}/F_{362}) without the drugs was approximately 1.2. Similar results were obtained several times.

Ca^{2+} Response in the Presence of both DTBHQ and MTBHQ

DTBHQ and MTBHQ, both of which are hydroquinones having one or two *tert*-butyl groups, had contrasting effects on the Ca^{2+} response of RBL-2H3 cells as shown above. Therefore, the effect of MTBHQ on the DTBHQ-induced response was investigated. As shown in Fig. 4, MTBHQ was

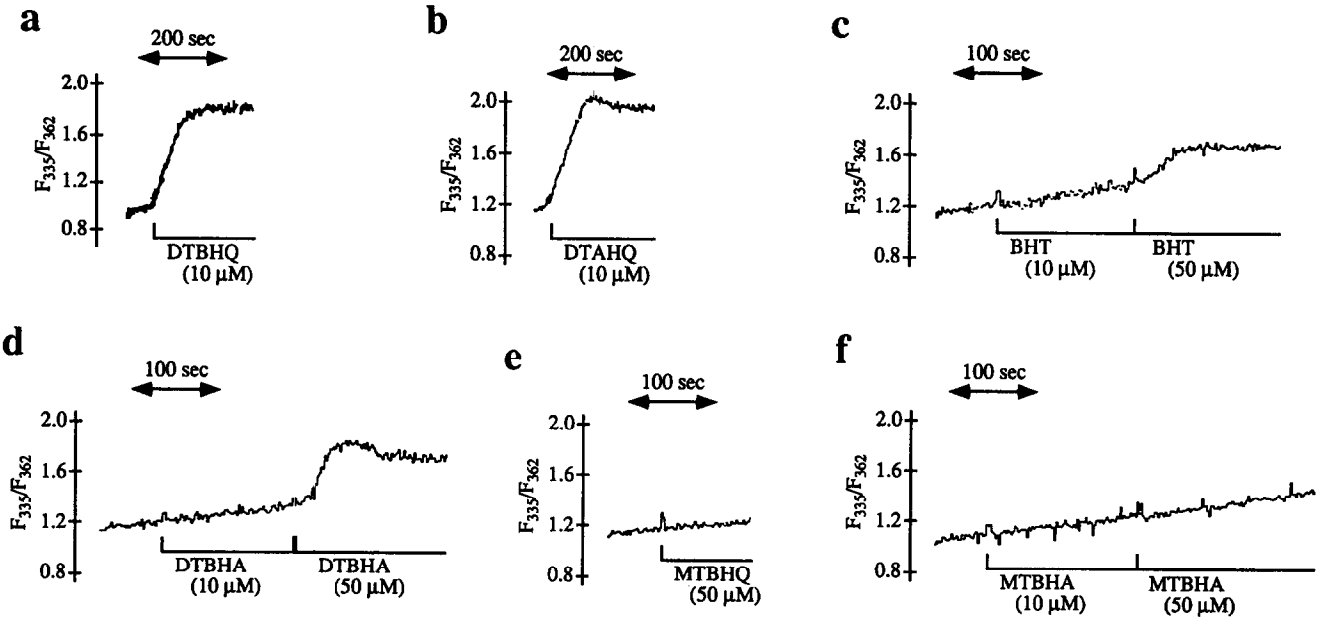


FIG. 2. Typical recordings of the effects of the six compounds on $[Ca^{2+}]_i$ in fura-2-loaded RBL-2H3 cells. (a) DTBHQ (10 μM); (b) DTAHQ (10 μM); (c) BHT (10 μM, 50 μM); (d) DTBHA (10 μM, 50 μM); (e) MTBHQ (50 μM); (f) MTBHA (10 μM, 50 μM). Similar results were obtained several times.

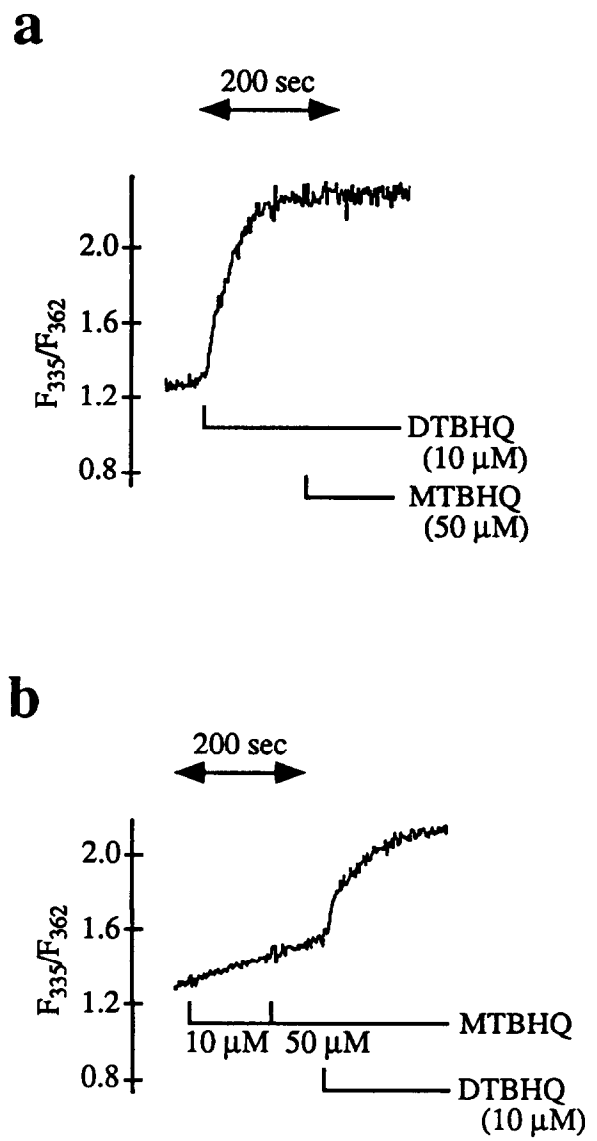


FIG. 4. Effect of MTBHQ on the DTBHQ-induced calcium response of RBL-2H3 cells. (a) MTBHQ (50 μM) was added 150 sec after the addition of DTBHQ (10 μM). (b) MTBHQ (10 μM, 50 μM) was added before the addition of DTBHQ (10 μM). Similar results were obtained several times.

inert, whether it was added before or after DTBHQ. Thus, MTBHQ was not antagonistic.

Effects of DTBHQ and DTAHQ on $[Ca^{2+}]_i$ in the Presence of Antigen

As shown in Fig. 5, the antigen, dinitrophenylated BSA (DNP_7-BSA) (10 μg/mL), induced a rapid and sustained increase in $[Ca^{2+}]_i$. In the presence of the antigen, addition of DTBHQ (10 μM) induced an additional increase in $[Ca^{2+}]_i$ (Fig. 5a), as previously reported [14]. DTAHQ (10 μM) also enhanced the Ca^{2+} response in the same manner as DTBHQ (Fig. 5b). The other compounds, BHT, DTBHA, MTBHQ, and MTBHA, did not have a significant additive effect even at 50 μM (data not shown).

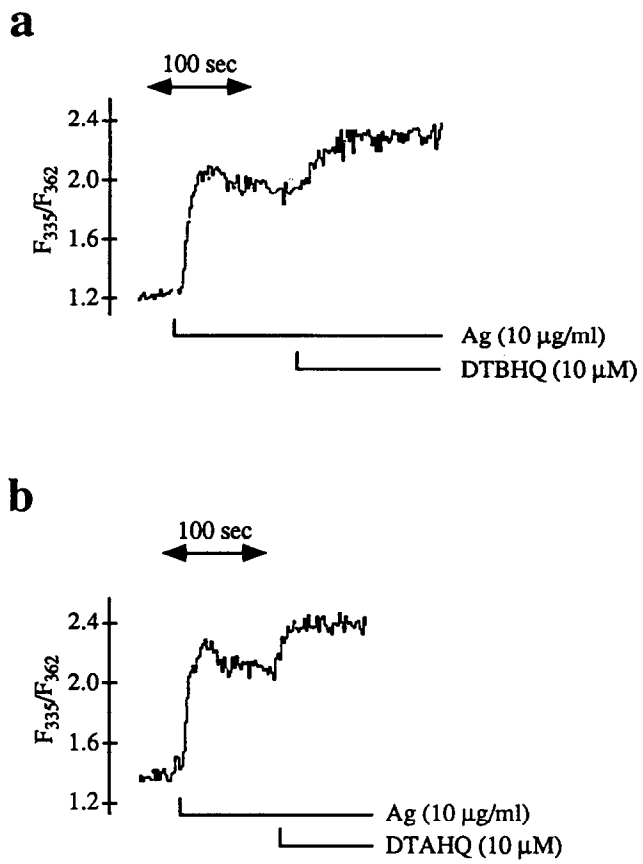


FIG. 5. Effects of DTBHQ and DTAHQ on the antigen-induced calcium response of RBL-2H3 cells. DNP_7-BSA (10 μg/mL) was added to cells preincubated with anti-DNP IgE, and then DTBHQ (10 μM) (a) or DTAHQ (10 μM) was added after 100 sec. Similar results were obtained several times.

Effects of RBL-2H3 Cells on Degranulation

The effects of the six hydroquinones and phenols on degranulation of RBL-2H3 cells were investigated. As an indication of degranulation, the activity of β-hexosaminidase released from the granules was measured [21].

Figure 6a shows the effect of each compound alone. DTAHQ caused the release of β-hexosaminidase to a similar extent as DTBHQ, but at a lower concentration than that of DTBHQ. None of the other compounds induced degranulation by themselves.

The effects of the compounds on antigen-induced degranulation are shown in Fig. 6b. Both DTAHQ and DTBHQ greatly increased antigen-induced β-hexosaminidase secretion at 10 μM. DTBHA also increased antigen-induced degranulation at higher concentrations. BHT slightly increased the response at 10 μM, but was rather inhibitory at 50 μM. The ED_{50} of DTBHQ, DTAHQ, and DTBHA were 1.8 μM, <1 μM, and 11 μM, respectively. MTBHQ and MTBHA showed no significant effect on antigen-induced degranulation.

As previously reported, DTBHQ acts synergistically with TPA to induce degranulation [14]. Therefore, we examined

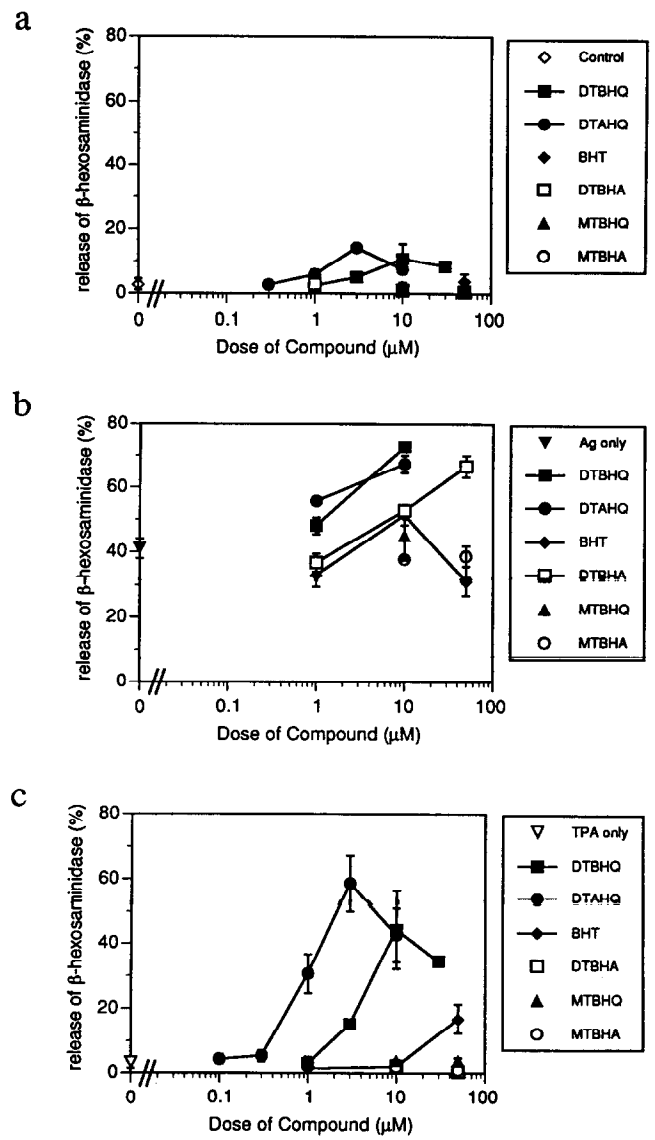


FIG. 6. Dose dependency of β-hexosaminidase release induced by DTBHQ, DTAHQ, BHT, DTBHA, MTBHQ, and MTBHA from RBL-2H3 cells. (a) compound alone; (b) with antigen; (c) with TPA. The cells were incubated with each compound and buffer, DNP_7-BSA (10 μg/mL), or TPA (10 ng/mL) for 40 min, and the activity of released β-hexosaminidase was determined. The total (released and residual) amounts of β-hexosaminidase were taken as 100%. Each bar represents the mean ± SD for data from 2 or 3 experiments done in duplicate.

the enhancing effect of these drugs in the presence of TPA (10 ng/mL). DTAHQ caused a marked increase in β-hexosaminidase release at 3 μM and BHT caused a slight increase at 50 μM, but TPA alone did not increase enzyme release (Fig. 6c). The other three compounds did not induce degranulation in combination with TPA, even at 50 μM. The ED_{50} of DTBHQ, DTAHQ, and BHT were 4.1 μM, 1.0 μM, and 22 μM, respectively.

As shown in Fig. 3, these compounds increased $[Ca^{2+}]_i$ in a dose-dependent manner. Also, in the presence of TPA, the compounds induced β-hexosaminidase release dose-dependently (Fig. 6c). Therefore, the relationship between $[Ca^{2+}]_i$ and β-hexosaminidase release obtained from the dose-response experiments was investigated. As shown in Fig. 7, the release of β-hexosaminidase in the presence of TPA was clearly dependent on $[Ca^{2+}]_i$. Higher Ca^{2+} responses caused by DTBHQ and DTAHQ led to greater degrees of degranulation, and the smaller Ca^{2+} response caused by BHT led to a smaller degree of degranulation. (In the resting state, $[Ca^{2+}]_i$ of RBL-2H3 cells was *ca.* 62 nM, and the spontaneous release of the enzyme was 2 ~ 3%.)

First Oxidation Potentials of Six Antioxidants

As with the antioxidant activity, the first oxidation potentials of six compounds were measured. The results are shown in Table 1. The oxidation potential of BHT, 1.09 V, was the highest (i.e. it was the weakest antioxidant among these compounds). The oxidation potentials of the other five compounds were in the range of 0.71 ~ 0.85 V. Thus, the oxidation potentials of these six antioxidants did not correlate with their Ca^{2+}-increasing activity.

DISCUSSION

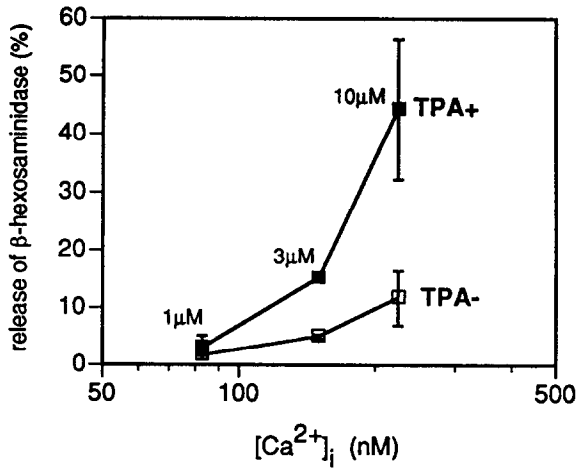
In this study, we have investigated the effects of five hydroquinones and phenols on the calcium response and degranulation of RBL-2H3 cells and compared their effects with those of DTBHQ.

It has been suggested that, in RBL-2H3 cells, antigen-induced histamine secretion is entirely dependent on elevation of intracellular Ca^{2+} [3]. Our results indicate that DTAHQ causes a significant increase in $[Ca^{2+}]_i$. However, the degree of degranulation was much smaller than that caused by antigen stimulation (Fig. 6a and b). These effects of DTAHQ are very similar to those of DTBHQ [14]. The four other compounds caused less or no increase in $[Ca^{2+}]_i$ and no degranulation of the cells by themselves. Thus, the elevation of $[Ca^{2+}]_i$ is necessary, but not sufficient, for the level of degranulation induced by antigen.

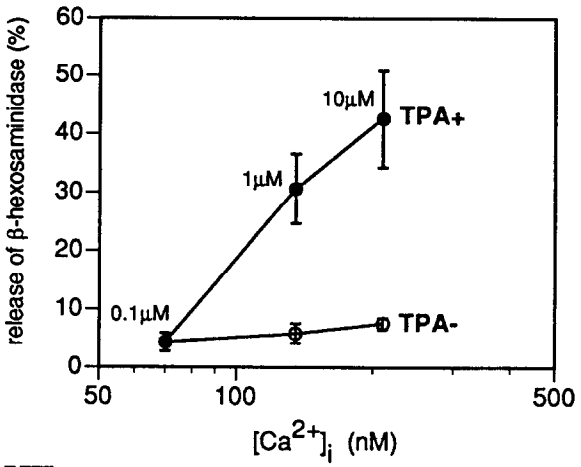
The protein kinase C activator TPA, which did not induce degranulation by itself, greatly enhanced the effects of DTAHQ and DTBHQ (Fig. 6c). The similar synergistic effect of phorbol ester and A23187 were observed in RBL-2H3 cells [22]. In human basophils as well, a low concentration (3 ng/mL) of TPA induces histamine release synergistically with A23187 [23]. In the case of antigen stimulation, the necessity of protein kinase C activation for degranulation is demonstrated by using protein kinase C inhibitors [19, 22]. Therefore, the result that the presence of both TPA and DTAHQ (or DTBHQ) leads to a high level of degranulation is consistent with the results obtained in the antigen stimulation.

As shown in Fig. 7, the abilities of all three compounds to induce the Ca^{2+} response and to release β-hexosaminidase in the presence of TPA correlated well. Below 80 nM of $[Ca^{2+}]_i$, the degree of degranulation in the presence of

a. DTBHQ



b. DTAHQ



c. BHT

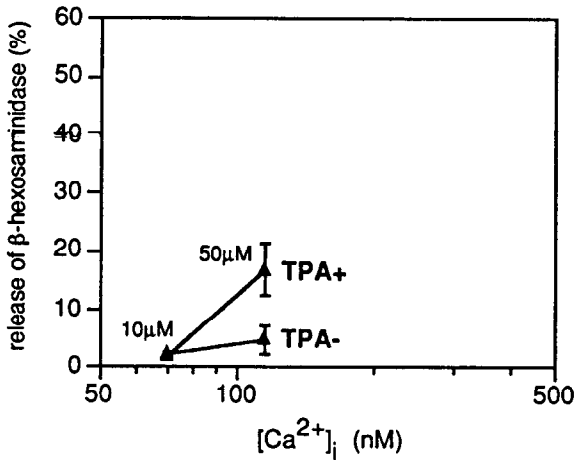


FIG. 7. Relationship between $[Ca^{2+}]_i$ and release of β-hexosaminidase induced by various concentrations of DTBHQ (a), DTAHQ (b), and BHT (c) in the presence (closed symbols) or absence (open symbols) of TPA. Antioxidant concentrations used are shown near the symbols. The total (released and residual amounts of β-hexosaminidase were taken as 100%. Each bar represents the mean ± SD for data from 2 or 3 experiments done in duplicate.

TABLE 1. First oxidation potentials of six antioxidants

Compound	First Oxidation Potential (V)
DTBHQ	0.73
DTAHQ	0.71
BHT	1.09
DTBHA	0.76
MTBHQ	0.78
MTBHA	0.85

The potentials were measured in acetonitrile containing 0.1 M tetraethylammonium perchlorate under N_2. Reference electrode: Ag/Ag^+, scan rate: 100 mV/s.

TPA was small. When higher Ca^{2+} responses were induced by the drugs, greater degrees of degranulation occurred. Thus, degranulation with TPA was dependent on the increase in $[Ca^{2+}]_i$.

Among the phenolic antioxidants, only BHT had the effect of enhancing degranulation with TPA (Fig. 6c), and DTBHA did not increase degranulation. BHA, but not BHT, is suggested to have some biological effects, including lipoxygenase inhibition [16]. Therefore, it is postulated that DTBHA might have an inhibitory effect on the phorbol ester-activated pathway in RBL-2H3 cells.

As shown in Fig. 3, DTAHQ, DTBHQ, and DTBHA induced $[Ca^{2+}]_i$ elevation. In addition, DTAHQ and DTBHQ were shown to increase the antigen-induced calcium response (Fig. 5). The calcium-increasing activity of these three compounds correlated well with their increased effect on antigen-induced degranulation (Fig. 6b). Therefore, the compounds are assumed to modulate the antigen-induced degranulation level by increasing $[Ca^{2+}]_i$.

The structural similarity of the effective compounds used suggests that they function through the same mechanism as DTBHQ (i.e. inhibition of Ca^{2+}-ATPase in the endoplasmic reticulum). It is very interesting that DTBHQ and DTAHQ were very effective but MTBHQ showed no effect, although they all have a 1,4-hydroquinone structure. In the cases of DTBHA and MTBHA, which are both 4-hydroxyanisole-type compounds, DTBHA was effective to some extent but MTBHA had no effect. All six compounds investigated in this study are widely used antioxidants. The oxidation potential of BHT, which induces a moderate Ca^{2+} response, was the highest (1.09 V), and the potentials of the other five compounds were all near 0.7 ~ 0.8 V (Table 1). Thus, there is no relationship between Ca^{2+} response and the antioxidant activity of the compounds. The differences in their structure (i.e. the number of *tert*-butyl or *tert*-amyl groups, which are very bulky), should determine their ability to induce the Ca^{2+} response. Further information about the structure-activity relationship should be helpful for understanding the mechanism of Ca^{2+}-ATPase inhibition.

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