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Effect of t-Butyl Hydroperoxide on Ca^{2+} Movement in PC12 Pheochromocytoma Cells

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Abstract

The effect of the oxidant t-butyl hydroperoxide on intracellular free levels of Ca^{2+} ($[Ca^{2+}]_i$) in PC12 pheochromocytoma cells was examined by using fura-2 as a fluorescent dye. t-Butyl hydroperoxide induced an increase in $[Ca^{2+}]_i$ in a concentration-dependent fashion between 50-250 μM with an EC50 of 100 μM. The $[Ca^{2+}]_i$ signal consisted of a slow rise and a sustained phase. The response was decreased by 65% by removal of extracellular Ca^{2+}. In Ca^{2+}-free medium, pretreatment with 1 μM thapsigargin (an endoplasmic reticulum Ca^{2+} pump inhibitor) abolished 150 μM t-butyl hydroperoxide-induced $[Ca^{2+}]_i$ increase, and conversely, pretreatment with t-butyl hydroperoxide abrogated thapsigargin-induced $[Ca^{2+}]_i$ increase. The 150 μM t-butyl hydroperoxide-induced $[Ca^{2+}]_i$ increase in Ca^{2+} medium was reduced by 42±5% by pretreatment with 0.1 μM nicardipine but not by 10 μM verapamil, nifedipine, nimodipine or diltiazem, or by 50 μM La^{3+} or Ni^{2+}. Pretreatment with 10 μM t-butyl hydroperoxide for 40 min did not affect 10 μM ATP-induced $[Ca^{2+}]_i$ increase. Together, the results show that t-butyl hydroperoxide induced significant $[Ca^{2+}]_i$ increase in PC12 cells by causing store Ca^{2+} release from the thapsigargin-sensitive endoplasmic reticulum pool in an inositol 1,4,5-trisphosphate-independent manner and by inducing Ca^{2+} influx via a nicardipine-sensitive pathway.

Key Words: Ca^{2+}; Ca^{2+} stores; fura-2; t-butyl hydroperoxide; PC12 cells; thapsigargin

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Introduction

Evidence supports the view that oxidative stress may play a crucial role in cellular abnormalities in many diseases and that the antioxidant therapy may prove beneficial in combating these problems (13). However, physiologically relevant concentrations of reactive oxygen species (ROS) can regulate many key molecular mechanisms that may be linked with important cell functions (26, 28). Under oxidative stress—for example, ischaemia-reoxygenation injury to cells—mitochondria form superoxide, which in turn is converted to hydrogen peroxide and the potent reactive species, hydroxyl radical (10). These deleterious effects of ROS are due to their interaction with various ion transport proteins underlying the transmembrane signal transduction, such as channels, ion pumps, ion exchangers, and ion cotransporters. Alterations in the ion transport mechanisms lead to changes in a second messenger system, primarily Ca^{2+} homeostasis, which further augment the abnormal electrical activity and distortion of signal transduction, causing cell dysfunction, which underlies pathological conditions (19).

Oxidant stress has been shown to alter Ca^{2+} homeostasis (15, 16). t-Butyl hydroperoxide is one of the many drugs that have been used to induce oxidative stress (14). t-Butyl hydroperoxide was found to cause death in cells (1, 18). In hepatocytes, t-butyl hydroperoxide was shown to cause an increase in cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) mediated by release of store Ca^{2+} (3). t-Butyl hydroperoxide was also found to cause an increase in mitochondrial permeability transition in hepatocytes (7, 8).

An increase in $[Ca^{2+}]_i$ is a key signal for many cell functions (6, 12). A $[Ca^{2+}]_i$ increase may occur as a result of release of Ca^{2+} from intracellular stores and/or an entry of Ca^{2+} from extracellular space. The inositol 1,4,5-trisphosphate-sensitive Ca^{2+} store is an important intracellular Ca^{2+} pool which actively discharges Ca^{2+} into cytosol when the inositol 1,4,5-trisphosphate receptors on these stores bind cytosolic inositol 1,4,5-trisphosphate (4, 5). In many cell types, this Ca^{2+} mobilization may cause Ca^{2+} influx across plasma membrane via the process of capacitative Ca^{2+} entry (23). Prolonged elevations in $[Ca^{2+}]_i$ or abnormal regulations of $[Ca^{2+}]_i$ are known to lead to cell injury and apoptosis (6, 12).

Previous evidence suggests that t-butyl-hydroperoxide could elevate resting mitochondrial membrane potential (20) and cause DNA strand scission in PC12 cells (27). The effect of t-butyl-hydroperoxide on Ca^{2+} handling in PC12 cells was unclear. The present study was aimed to explore the effect of t-butyl-hydroperoxide on $[Ca^{2+}]_i$ in PC12 cells. By using fura-2 as a fluorescent Ca^{2+} probe, this study shows that t-butyl-hydroperoxide induced a significant increase in $[Ca^{2+}]_i$. The concentration-response relationship was established, and the underlying mechanism of the $[Ca^{2+}]_i$ increase was evaluated.

Materials and Methods

Cell Culture

PC12 cells obtained from American Type Culture Collection were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 g/ml streptomycin. The cells were kept at 37°C in 5% CO_2-containing humidified air. The PC12 cells were undifferentiated (i.e., resembled adrenal chromaffin cells rather than differentiated with nerve growth factor to resemble sympathetic neurons).

Solutions

Ca^{2+}-containing medium (pH 7.4) contained (in mM): NaCl 140; KCl 5; $MgCl_2$ 1; $CaCl_2$ 2; Hepes 10; glucose 5. Ca^{2+}-free medium contained no Ca^{2+} plus 1 mM EGTA. Drugs were dissolved in water, ethanol or dimethyl sulfoxide as concentrated solutions which were diluted before use. The concentration of solvents in the final experimental solution did not exceed 1% and did not alter basal $[Ca^{2+}]_i$ (n = 4).

Fluorescence Measurement

Trypsinized cells (10^6/ml) were allowed to recover in the medium for 1 hr before being loaded with 2 μM fura-2/AM for 30 min at 25°C in the same medium. The cells were washed and resuspended in Ca^{2+} medium. Fura-2 fluorecence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring. The cuvette contained 1 ml of medium and 0.5 million cells. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer (Shimadzu Corp., Kyoto, Japan) by continuously recording excitation signals at 340 and 380 nm and emission signal at 510 nm at 1-s intervals. Maximum and minimum fluorescence values were obtained by addition of 0.1% Triton X-100 (plus 10 mM $CaCl_2$) and 20 mM EGTA sequentially at the end of each experiment. $[Ca^{2+}]_i$ was calculated as described previously assuming a K_d of 155 nM (17).

Reagents

The reagents for cell culture were from Gibco (Grand Island, NY, USA). Fura-2/AM was from Molecular Probes (Eugene, OR, USA). U73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)

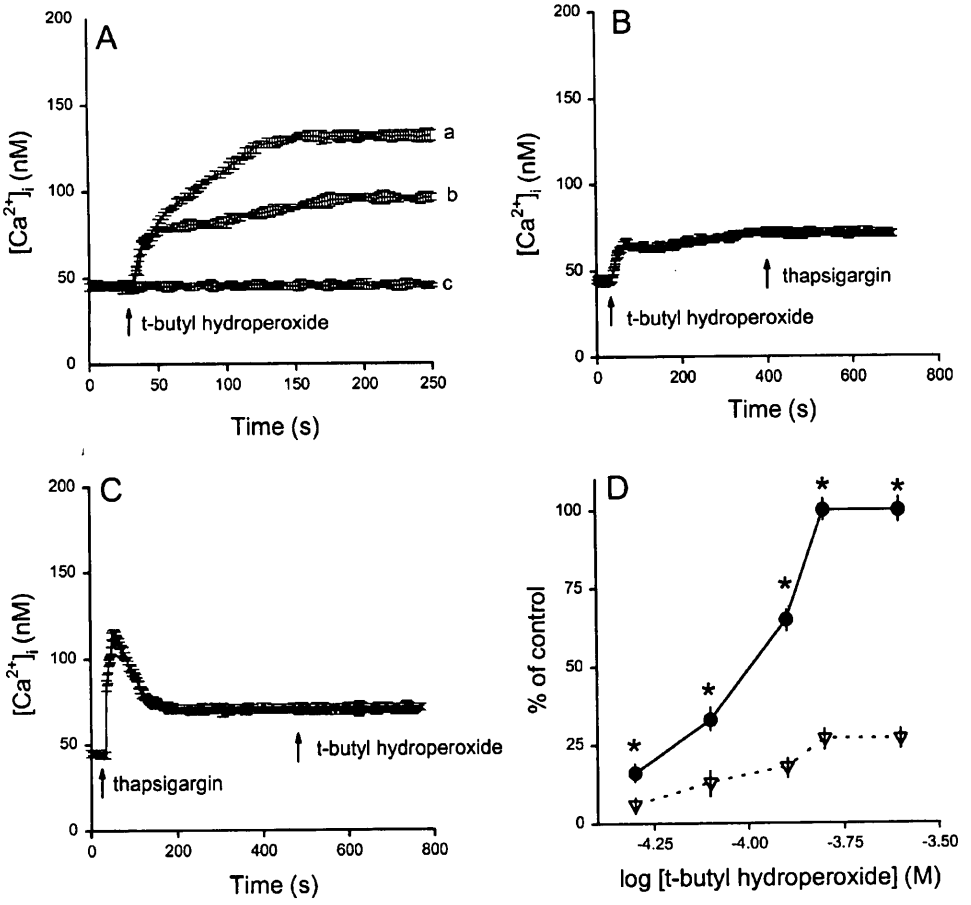

Fig. 1. Effect of t-butyl hydroperoxide on $[Ca^{2+}]_i$ in PC12 cells. (A) Concentration-dependent effects of t-butyl hydroperoxide in Ca^{2+} medium. The concentration of t-butyl hydroperoxide was 150 μM in *trace a*, 50 μM in *trace b*, and 10 μM in *trace c*. The drug was added at 30 s. Control ($[Ca^{2+}]_i$ in the absence of t-butyl hydroperoxide) was identical to trace c, and was thus not shown. (B) In Ca^{2+}-free medium, 150 μM t-butyl hydroperoxide was added at 30 s followed by adding 1 μM thapsigargin at 400 s. (C) In Ca^{2+}-free medium, 1 μM thapsigargin was added at 30 s followed by addition of 150 μM tBHP at 500 s. (D) Concentration-response plots of t-butyl hydroperoxide-induced responses in the presence (*filled circles*) or absence (*open triangles*) of Ca^{2+}. The y axis is the percentage of control. Control was the net maximum $[Ca^{2+}]_i$ value of 250 mM t-butyl hydroperoxide-induced $[Ca^{2+}]_i$ increase in Ca^{2+} medium. The data were means±SEM of 4-6 replicates. *P < 0.05 between *filled circles* and open triangles.

amino)hexyl)-1H-pyrrole-2,5-dione) and U73343 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl) amino)hexyl)-2,5-pyrrolidine-dione) were from Biomol (Plymouth Meeting, PA, USA). The other reagents were from Sigma (St. Louis, MO, USA).

Statistics

The data were reported as means±SEM of 4-6 replicates. Statistical comparisons were determined by using Student's *t* test, and significance was accepted when $P < 0.05$.

Results

Effect of t-butyl Hydroperoxide on $[Ca^{2+}]_i$

In Ca^{2+}-containing medium, t-butyl hydroperoxide at concentrations between 50-250 μM increased $[Ca^{2+}]_i$ in a concentration-dependent manner. Figure 1A shows the $[Ca^{2+}]_i$ increases induced by 150 μM (trace a) and 50 μM (trace b) t-butyl hydroperoxide. At a concentration of 10 μM, t-butyl hydroperoxide had no effect on $[Ca^{2+}]_i$ (trace c). The basal $[Ca^{2+}]_i$ was 49±3 nM (n = 6). Over a time period of 250 s, the $[Ca^{2+}]_i$ signal induced by 150 μM t-butyl hydroperoxide consisted of an immediate rise which reached a net (baseline subtracted) maximum value of 91±3 nM (n = 6), followed by a plateau. The Ca^{2+} signal saturated at 250 μM t-butyl hydroperoxide because 500 μM t-butyl hydroperoxide did not induce a greater response (n = 5; data not shown). Figure 1D (filled circles) shows the concentration-response curve of the t-butyl hydroperoxide response. The curve suggests an EC_{50} value of about 100 μM in Ca^{2+} medium.

Effect of Removing Extracellular Ca^{2+} on t-butyl Hydroperoxide-induced $[Ca2^{2+}]_i$ Increase

Experiments were performed to evaluate the relative contribution of intracellular Ca^{2+} release and extracellular Ca^{2+} entry in the t-butyl hydroperoxide response. In Ca^{2+}-free medium (no added Ca^{2+} plus 1 mM EGTA to chelate residual Ca^{2+}), t-butyl hydroperoxide increased $[Ca^{2+}]_i$ in a concentration-dependent manner. Figure 1B shows 150 μM t-butyl hydroperoxide-induced response. The $[Ca^{2+}]_i$ increase had a net maximum value of 24±2 nM (n = 5). The concentration-response curve of t-butyl hydroperoxide-induced $[Ca^{2+}]_i$ increases in Ca^{2+}-free medium is shown in Figure 1D (open triangles). The data suggest that Ca^{2+} removal inhibited the $[Ca^{2+}]_i$ increases by a range of 6510% for the various concentrations of t-butyl hydroperoxide tested.

The Intracellular Source of t-butyl Hydroperoxide-induced $[Ca^{2+}]_i$ Increase

Figure 1B shows that in Ca^{2+}-free medium, after incubation with 150 μM t-butyl hydroperoxide for 6 min, addition of 1 μM thapsigargin, an endoplasmic reticulum Ca^{2+} pump inhibitor (30), barely induced a $[Ca^{2+}]_i$ increase (n = 6). Figure 1C shows that thapsigargin (1 μM) induced a $[Ca^{2+}]_i$ increase with a net maximum value of 713 nM (n = 4). The maximum $[Ca^{2+}]_i$ increase was followed by a slow decay and a sustained phase with a net $[Ca^{2+}]_i$ value of 20±2 nM. Subsequently added t-butyl hydroperoxide (150 μM) failed to increase $[Ca^{2+}]_i$ (n = 4).

Effect of Ca^{2+} Entry Blockers on t-butyl Hydroperoxide-induced $[Ca^{2+}]_i$ Increase

In Ca^{2+}-containing medium, pretreatment with 50 μM La^{3+}, or 10 μM Ni^{2+}, nifedipine, nimodipine, verapamil, and diltiazem did not affect 150 μM t-butyl hydroperoxide-induced $[Ca^{2+}]_i$ increase (n = 4; data not shown). The only effective blocker was nicardipine (0.1 μM) which inhibited the t-butyl hydroperoxide response by 423% (n = 6; $P < 0.05$) in the net maximum value (Figure 2).

Role of Phospholipase C in t-butyl Hydroperoxide-induced Ca^{2+} Release

Pretreatment for several min with the phospholipase C inhibitor U73122 at a concentration of 2 μM has been shown to effectively block inositol 1,4, 5-trisphosphate formation in cells without causing much alteration in $[Ca^{2+}]_i$ (31). Our data suggest that incubation with 2 μM U73122 for 3 min did not affect basal $[Ca^{2+}]_i$ but abolished 10 μM ATP-induced

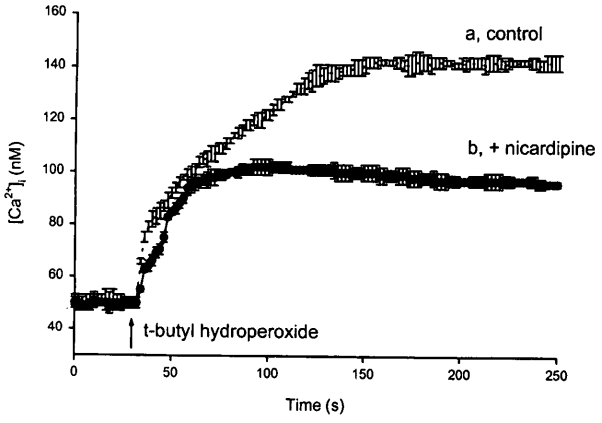

Fig. 2. Effect of nicardipine on t-butyl hydroperoxide-induced $[Ca^{2+}]$ increase. *Trace a*: control 150 μM t-butyl hydroperoxide response. *Trace b*: 0.1 μM nicardipine was added at time zero followed by addition of 150 μM t-butyl hydroperoxide at 30 s. The data were means±SEM of 4-6 replicates.

$[Ca^{2+}]_i$ increase; and conversely, 10 μM U73343, an inactive analogue of U73122, had no inhibition on the ATP response (n = 4; data not shown). This suggests that U73122 effectively inhibited phospholipase C activity. However, treatment with U73122 for 9 min did not alter 150 μM t-butyl hydroperoxide-induced $[Ca^{2+}]_i$ increase (n = 4; data not shown).

Effect of a Low Concentration of t-butyl Hydroperoxide on ATP-induced $[Ca^{2+}]_i$ Increase

In order to know whether long-term exposure of t-butyl hydroperoxide could alter the $[Ca^{2+}]_i$ increase induced by a physiological agonist, the effect of incubation with 10 μM t-butyl hydroperoxide for 40 min in Ca^{2+}-containing medium on 10 μM ATP-induced $[Ca^{2+}]_i$ increase was examined. ATP induced a $[Ca^{2+}]_i$ increase with a net maximum value of 223±4 nM. Pretreatment with t-butyl hydroperoxide did not alter the ATP response (n = 4; data not shown). As stated in Figure 1A, 10 μM t-butyl hydroperoxide did not alter basal $[Ca^{2+}]_i$.

Discussion

This study is the first to show that t-butyl hydroperoxide induced an increase in $[Ca^{2+}]_i$ in PC12 cells. t-Butyl hydroperoxide increased $[Ca^{2+}]_i$ by causing both intracellular Ca^{2+} release and extracellular Ca^{2+} entry because the Ca^{2+} signals obtained in Ca^{2+}-containing medium were partly inhibited by Ca^{2+} removal. The rising and sustained phases of the $[Ca^{2+}]_i$ increase were both reduced by Ca^{2+} removal, suggesting that the $[Ca^{2+}]_i$ increase involves Ca^{2+} entry and Ca^{2+} release throughout the five min of measurement.

It appears that t-butyl hydroperoxide-induced Ca^{2+} influx involves a nicardipine-sensitive pathway. It is interesting that other dihydropyridines including nifedipine and nimodipine did not have similar effect. This may be because that in the micromolar range, dihydropyridines have multiple sites of action (32) and there are differences in the allosteric modulation of Ca^{2+} channels by different channel blockers (9, 22). Furthermore, note that nicardipine decreased the t-butyl hydroperoxide response by 42±3%, whereas the experiments in low Ca^{2+} medium suggest that approximately 65% of the Ca^{2+} came from extracellular sources; thus, nicardipine-sensitive pathways are probably not the only route for Ca^{2+} entry. Nifedipine and verapamil have been shown to effectively inhibit L-type Ca^{2+} channels in PC12 cells (29). However, evidence shows that some drugs can induce Ca^{2+} influx in PC12 cells via pathways insensitive to nifedipine, verapamil and diltiazem (11). The lack of effect of La^{3+} and Ni^{2+} could be because that these metals not only inhibited Ca^{2+} influx but Ca^{2+} efflux too. In chromaffin cells, La^{3+} was shown to potentiate agonists-induced $[Ca^{2+}]_i$ increases and catecholamine secretion by inhibiting Ca^{2+} efflux (21). Since t-butyl hydroperoxide induced intracellular Ca^{2+} release, blockade of Ca^{2+} efflux would raise $[Ca^{2+}]_i$ and mask the inhibitory effect of La^{3+} on Ca^{2+} influx.

The results indicate that t-butyl hydroperoxide evoked intracellular Ca^{2+} release mainly by discharging Ca^{2+} from the thapsigargin-sensitive stores, because in Ca^{2+}-free medium, pretreatment with 150 μM t-butyl hydroperoxide prevented 1 μM thapsigargin from releasing more Ca^{2+}; and conversely, pretreatment with thapsigargin completely depleted the Ca^{2+} pool releasable by t-butyl hydroperoxide.

A question was how t-butyl hydroperoxide causes Ca^{2+} release. Our data suggest that t-butyl hydroperoxide-induced Ca^{2+} release did not depend on the activity of phospholipase C. t-Butyl hydroperoxide may act by inhibition of the the endoplasmic reticulum Ca^{2+} pump as demonstrated in red blood cells (25).

At a concentration that does not increase the basal $[Ca^{2+}]_i$, t-butyl hydroperoxide did not alter ATP-induced $[Ca^{2+}]_i$ increases. This suggests that t-butyl hydroperoxide may only be able to alter Ca^{2+} signaling at a higher concentration range.

Together, this study shows that t-butyl hydroperoxide causes a significant increase in $[Ca^{2+}]_i$ in PC12 cells. The data suggest that t-butyl hydroperoxide increases $[Ca^{2+}]_i$ in a concentration-dependent manner by releasing Ca^{2+} from thapsigargin-sensitive stores in a phospholipase C-independent manner, and also by inducing nicardipine-sensitive Ca^{2+} influx. Due to the general importance of a $[Ca^{2+}]_i$ increase in cell function, these results may help to explain the diverse *in vivo* and *in vitro* effects of t-butyl hydroperoxide. A recent paper has shown that H_2O_2 induced a $[Ca^{2+}]_i$ increase in PC12 cells and the effect was attributed to store Ca^{2+} release from mitochondria and external Ca^{2+} influx through verapamil- and tetrodotoxin-sensitive pathways (33). Thus, different oxidizing agents may alter Ca^{2+} signaling in the same cell type in a different manner.

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