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2,5-Di-(*tert*-butyl)-1,4-benzohydroquinone mobilizes inositol 1,4,5-trisphosphate-sensitive and -insensitive Ca^{2+} stores

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In permeabilized rat hepatocytes a maximal concentration (25 μM) of 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone (tBuBHQ) mobilized 70% of sequestered Ca^{2+} and a half-maximal effect was produced by 1.7 μM tBuBHQ. Inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) stimulated release of about 40% of the intracellular Ca^{2+} stores. Combined applications of a range of tBuBHQ concentrations with a maximal concentration of Ins(1,4,5)P_3 demonstrated that tBuBHQ has slight selectivity for the Ca^{2+} transport process of the Ins(1,4,5)P_3-sensitive stores. We conclude that the Ins(1,4,5)P_3-sensitive stores are a subset of those sensitive to tBuBHQ and that the latter is therefore unlikely to prove useful as a tool to discriminate Ins(1,4,5)P_3-sensitive and -insensitive Ca^{2+} stores though it may provide opportunities to design more selective agents.

Inositol 1,4,5-trisphosphate; Intracellular Ca^{2+} store; 2,5-Di-(*tert*-butyl)-1,4-benzohydroquinone

1. INTRODUCTION

Inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3), formed after activation of Ca^{2+}-mobilizing receptors, binds to specific intracellular receptors and thereby stimulates Ca^{2+} release from intracellular stores causing an increase in cytoplasmic [Ca^{2+}]. A sustained increase in cytoplasmic [Ca^{2+}]depends upon Ca^{2+} influx from the extracellular space [1]. Measurements on single cells reveal additional complexities because the intracellular Ca^{2+} signal appears to be precisely organised in both space and time with different areas of the cell responding differently and with signals often taking the form of a complex series of spikes [2,3]. Ins(1,4,5)P_3 appears to play a part, directly or indirectly, in regulating Ca^{2+} entry [4] and in controlling the more complex features of the Ca^{2+} signal [2,3]. A feature of many models proposed to account for these actions of Ins(1,4,5)P_3 is regulated communication between discrete intracellular Ca^{2+} stores, only a fraction of which are sensitive to Ins(1,4,5)P_3 [5]. However, neither the biochemical characteristics nor the anatomical identity of the Ins(1,4,5)P_3-sensitive Ca^{2+} stores are known [6]. Pharmacological tools that distinguish the different stores would clearly be useful.

Thapsigargin has been used to empty intracellular Ca^{2+} stores because it specifically inhibits the Ca^{2+}-ATPase of the stores without affecting plasma membrane Ca^{2+} transport, but it does not usually discriminate between Ins(1,4,5)P_3-sensitive and -insensitive stores [7]. 2,5-Di-(*tert*-butyl)-1,4-benzohydroquinone (tBuBHQ), however, has been claimed to selectively mobilize the same intracellular Ca^{2+} stores as Ins(1,4,5)P_3 [8]. Here we demonstrate that although tBuBHQ has some selectivity for the Ca^{2+} transport system of Ins(1,4,5)P_3-sensitive stores, the selectivity is weak and tBuBHQ mobilizes substantially more Ca^{2+} than does a maximally effective concentration of Ins(1,4,5)P_3.

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2. MATERIALS AND METHODS

The methods used to prepare and permeabilize rat hepatocytes and to measure $^{45}Ca^{2+}$ fluxes in the permeabilized cells have been described in detail in earlier publications [9,10]. Briefly, isolated hepatocytes were permeabilized by incubation with saponin (75 μg/ml, 10 min, 37°C) in a Ca^{2+}-free cytosol-like medium (140 mM KCl, 20 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 20 mM Pipes, pH 6.8), washed and resuspended in the same medium with $CaCl_2$ added to give a final free [Ca^{2+}] of 120 nM. Cells were loaded to steady-state with $^{45}Ca^{2+}$ (2 μCi/ml) in the presence of mitochondrial inhibitors (10 μM oligomycin and 10 μM antimycin) and ATP (1.5 mM). The $^{45}Ca^{2+}$ contents of cells before and after additions were measured by rapid filtration of the samples and then expressed relative to the ATP-dependent $^{45}Ca^{2+}$ content of the cells.

Ca^{2+} fluxes were measured with fluo-3 at 37°C by resuspending permeabilized cells (10^7/ml) in nominally Ca^{2+}-free cytosolic medium (without EGTA) in the presence of mitochondrial inhibitors (oligomycin 10 μM; antimycin 10 μM). Fluorescence (λ_{ex} = 503 nm, λ_{em} = 530 nm) was measured with a Perkin-Elmer LS50 luminescence spectrometer. Autofluorescence was recorded before addition of fluo-3 free acid (2 μM) and Ca^{2+} uptake was then initiated by addition of ATP (1.5 mM). After the permeabilized cells had reduced the free [Ca^{2+}] to a new steady-state, tBuBHQ or Ins(1,4,5)P_3 was added and ionomycin (1 μM) was subsequently added to release all sequestered Ca^{2+}. Traces were calibrated by addition of EGTA (2 μM) to obtain F_{min} followed by Ca^{2+} (15 μM) to obtain F_{max} and the [Ca^{2+}] then computed from:

$$[Ca^{2+}] = K_d(F-F_{min})/(F_{max}-F);\ K_d = 864\ \text{nM}\ [11].$$

Materials were from the suppliers listed in earlier publications [9]. Fluo-3 free acid was from Molecular Probes and tBuBHQ was from Aldrich.

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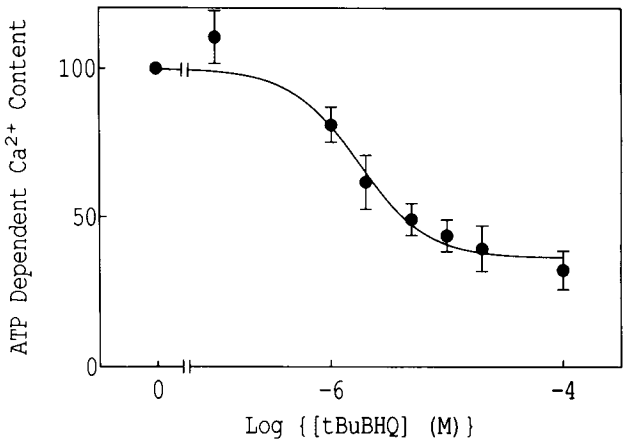

Fig. 1. Effect of tBuBHQ on $^{45}Ca^{2+}$ content of intracellular stores. ATP-dependent $^{45}Ca^{2+}$ content is shown after incubation for 5 min with tBuBHQ (mean ± SE, $n=7$).

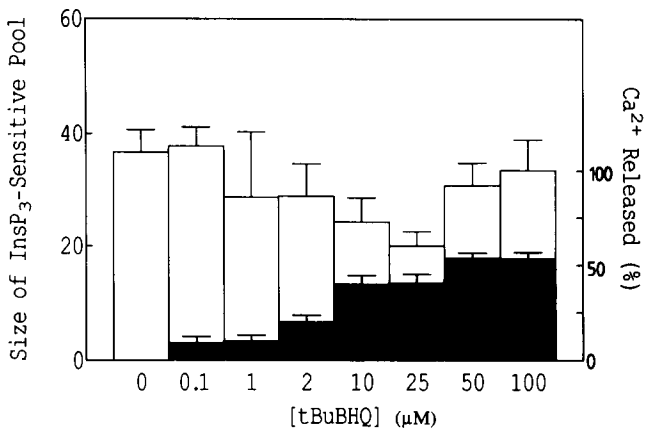

Fig. 2. Effect of tBuBHQ on the size of Ins(1,4,5)P_3-sensitive $^{45}Ca^{2+}$ stores. Cells were incubated with various concentrations of tBuBHQ (0–100 μM) for 2 min prior to addition of a maximal concentration of Ins(1,4,5)P_3 (10 μM) for 30 s. Shaded bars show $^{45}Ca^{2+}$ released by tBuBHQ alone (% of control; mean ± SE, $n=4$–6). Open bars show the amount of $^{45}Ca^{2+}$ released by Ins(1,4,5)P_3 expressed as a fraction of the $^{45}Ca^{2+}$ content remaining after the 2 min tBuBHQ treatment. Cells appear less sensitive to tBuBHQ in this figure (compare Fig. 1) only because they were exposed to tBuBHQ for a shorter period (2 min rather than 5 min).

3. RESULTS AND DISCUSSION

Addition of tBuBHQ to permeabilized hepatocytes loaded to steady-state with $^{45}Ca^{2+}$ (4.4 ± 0.4 nmol/mg protein) caused a concentration-dependent decrease in their $^{45}Ca^{2+}$ content (Fig. 1). A maximal effect, release of 68 ± 6% of the Ca^{2+} accumulated, was evoked by 25 μM tBuBHQ and a half-maximal effect occurred with 1.7 μM. These results, both the concentration-dependence and the inability of even supramaximal concentrations of tBuBHQ to fully empty the Ca^{2+} stores are the same as those reported by Kass et al. [8]. We have also confirmed in permeabilized cells results that they obtained in microsomes showing that unidirectional $^{45}Ca^{2+}$ efflux from preloaded stores is unaffected by tBuBHQ (results not shown). The effect of tBuBHQ is therefore to inhibit Ca^{2+} sequestration by the stores and not to stimulate a Ca^{2+} efflux pathway suggesting that the inhibitory effects of tBuBHQ on microsomal Ca^{2+}-ATPase [12] probably underlie its effects on intracellular Ca^{2+} stores.

A maximal concentration of Ins(1,4,5)P_3 (10 μM) stimulated release of 37 ± 4% of the $^{45}Ca^{2+}$ accumulated by the stores (Fig. 2), the remainder was rapidly released after addition of ionomycin. This is consistent with our earlier work [9] and many earlier reports [13–15]. By contrast, Kass et al. [8] reported that 80–90% of the stores were released by Ins(1,4,5)P_3. Our preparation, where a substantial fraction of the stores are insensitive to Ins(1,4,5)P_3, therefore provides a better opportunity to assess the selectivity of tBuBHQ for the Ins(1,4,5)P_3-sensitive and -insensitive stores. It is noteworthy that GTP has been reported to regulate the size of Ins(1,4,5)P_3-sensitive Ca^{2+} stores [13] and this may account for their variable size in different studies (25–90%) [8,9,13].

Earlier experiments [9] and the results shown in Fig. 3A demonstrate that the net Ca^{2+} release stimulated by Ins(1,4,5)P_3 is complete within 10 s. We therefore examined the selectivity of tBuBHQ for the Ins(1,4,5)P_3-sensitive stores by measuring the fraction of sequestered $^{45}Ca^{2+}$ that could be released by a maximal concentration of Ins(1,4,5)P_3 (10 μM for 30 s) after brief (2 min) tBuBHQ treatment. Under these conditions if tBuBHQ were to selectively inhibit Ca^{2+} sequestration into Ins(1,4,5)P_3-sensitive stores, we would expect that as the concentration of tBuBHQ is increased a progressively smaller fraction of the remaining stores would be released by a maximal concentration of Ins(1,4,5)P_3. The results (Fig. 2) demonstrate that within a narrow range of tBuBHQ concentrations (10–25 μM) there is a significant reduction (from 37 ± 4% to 20 ± 2%) in the fraction of sequestered Ca^{2+} released by Ins(1,4,5)P_3. At higher concentration of tBuBHQ (50–100 μM) Ca^{2+} sequestration by Ins(1,4,5)P_3-sensitive and -insensitive stores seems to be similarly inhibited because although the total Ca^{2+} content of the stores is substantially reduced (by 54 ± 3%) the fraction released by Ins(1,4,5)P_3 is similar in control (37 ± 4%) and tBuBHQ-treated (33 ± 5%) cells. These results suggest that tBuBHQ has some selectivity for Ins(1,4,5)P_3-sensitive stores, but only over a narrow concentration range.

The earlier study of tBuBHQ [8] used quin-2 to monitor the medium surrounding the permeabilized cells, but its high affinity for Ca^{2+} ($K_d=115$ nM) and low fluorescence intensity make it less sensitive to the observed changes in free [Ca^{2+}] (100–500 nM) than fluo-3 which is more fluorescent and has lower affinity for Ca^{2+} ($K_d=864$ nM at 37°C) [11]. When ATP was added to permeabilized cells in the presence of fluo-3

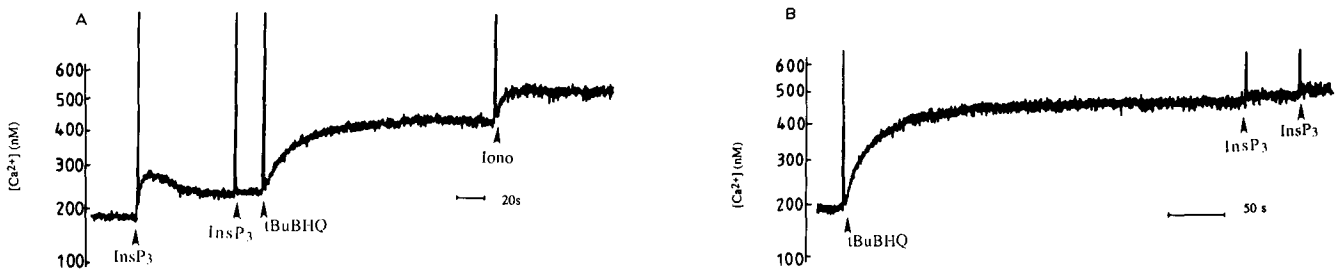

Fig. 3. Effects of Ins(1,4,5)P_3 and tBuBHQ monitored with fluo-3. The effects of sequential additions of Ins(1,4,5)P_3 (10 μM), tBuBHQ (25 μM), and ionomycin (1 μM) (Iono) are shown after the permeabilized cells have already reduced the free [Ca^{2+}] from about 400 to 200 nM following addition of ATP. Results are typical of at least 4 separate experiments.

they sequestered Ca^{2+} and reduced the free [Ca^{2+}] of the medium from about 400 nM to about 200 nM (Fig. 3). Addition of maximal concentration of Ins(1,4,5)P_3 (10 μM) stimulated release of 40% of the sequestered Ca^{2+} and subsequent addition of more Ins(1,4,5)P_3 evoked no further release. Half the remaining Ca^{2+} was released by a maximal concentration of tBuBHQ (25 μM) and ionomycin completely emptied the stores by stimulating release of the final 30% (Fig. 3A). Addition of tBuBHQ (25 μM) before Ins(1,4,5)P_3 abolished the response to the latter although the small amount of Ca^{2+} remaining in the stores was released by ionomycin (Fig. 3B). These results therefore confirm those obtained by $^{45}Ca^{2+}$ flux measurements.

There is convincing evidence that intracellular Ca^{2+} stores are heterogeneous [6] and in bovine adrenal chromaffin cells two immunologically distinct Ca^{2+}-ATPases with different subcellular distribution have been reported [16]. Our results suggest that tBuBHQ inhibits Ca^{2+} uptake by a fraction (about 80%) of intracellular stores that includes, but is not restricted to, the Ins(1,4,5)P_3-sensitive stores. The slight selectivity of low concentrations of tBuBHQ for Ins(1,4,5)P_3-sensitive stores may reflect the presence of distinct though closely related Ca^{2+}-ATPases in the Ins(1,4,5)P_3-sensitive and -insensitive stores. Although the selectivity of tBuBHQ is too weak for it to be useful it may provide opportunities to design more selective agents.

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