Comparison between the effects of the microsomal Ca^{2+} -translocase inhibitors thapsigargin and 2,5-di-(*t*-butyl)-1,4-benzohydroquinone on cellular calcium fluxes

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The effects of two inhibitors of the microsomal Ca^{2+} -ATPase, thapsigargin and 2,5-di-(*t*-butyl)-1,4-benzohydroquinone, were compared in hepatocytes and in a T-cell line (JURKAT). Both compounds mobilized the same intracellular Ca^{2+} pool, which contained the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive store, in hepatocytes and in JURKAT cells. The mobilization of the internal Ca^{2+} store with either compound activated Mn^{2+} entry in JURKAT cells, but not in hepatocytes. This suggests different properties of the bivalent-cation entry pathway between these cell types.

INTRODUCTION

The use of inhibitors of the microsomal Ca^{2+} -ATPase has proved to be very valuable in studies of the hormonal regulation of Ca^{2+} homeostasis in non-excitable cells. One of these inhibitors, thapsigargin, a sesquiterpene lactone tumour promoter [1], has been shown in several different cell types (including platelets, lymphocytes, neutrophils, macrophages, hepatocytes, adrenal chromaffin and parotid acinar cells [1]), to cause the release of the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} store, in the absence of inositol phosphate production [2–5]. However, thapsigargin also appeared to deplete an intracellular Ca^{2+} pool not mobilized by agonists [1,6,7]. Of considerable interest is the observation that thapsigargin reproduces the effects of agonists on Ca^{2+} entry in several, but not all, cell types tested, thereby providing strong support for the capacitative-entry hypothesis postulated by Putney and co-workers [4,8,9].

We have previously shown that another of the liver microsomal Ca^{2+} translocase, 2,5-di-(*t*-butyl)-1,4-benzohydroquinone (tBuBHQ), releases Ca^{2+} from the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} pool in isolated hepatocytes, without itself causing accumulation of inositol phosphates [10–12]. In this cell type, the tBuBHQ- and agonist-sensitive Ca^{2+} pools appeared to be identical. However, emptying of the agonist-sensitive Ca^{2+} pool by tBuBHQ did not stimulate Ca^{2+} influx into hepatocytes, as assessed by the Mn^{2+} -entry technique [11,13]. These seemingly contrasting results for Ca^{2+} fluxes in different cell types could be attributed to differences in the cellular effects of the inhibitors. In the present paper, the effects of thapsigargin and tBuBHQ, and those of Ca^{2+} -mobilizing agonists, were directly compared by using two cell types, isolated rat hepatocytes and a human leukaemic T-cell clone (JURKAT).

MATERIALS AND METHODS

Materials

Fura-2 acetoxymethyl ester and $[\text{Arg}^8]$ vasopressin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Collagenase (grade II) was purchased from Boehringer, Mannheim, Germany. tBuBHQ and thapsigargin were from EGA Chemie (Steinheim, Germany) and GIBCO BRL (Gaithersburg, MD, U.S.A.) respectively. OKT3 hybridoma was from American Type Tissue Culture (Rockville, MD, U.S.A.), and the antibodies

were purified from hybridoma supernatant by using Protein A-Sepharose. RPMI 1640 medium and fetal-calf serum were from GIBCO BRL.

Isolation of hepatocytes and maintenance of JURKAT cells

Hepatocytes were isolated from male Wistar rats (180–230 g, fed *ad libitum*) as previously described [14]. JURKAT cells (T-cell leukaemia) were maintained in continuous suspension culture in RPMI 1640 medium supplemented with 7.5 % (v/v) fetal-calf serum and antibiotics (100 units of penicillin/ml and 100 μg of streptomycin/ml) in a humidified incubator (37 °C) under an atmosphere of 5 % CO_2 in air.

Measurement of cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and Mn^{2+} influx in hepatocytes and JURKAT cells

Freshly isolated hepatocytes and JURKAT cells were loaded with fura-2, and measurements of $[\text{Ca}^{2+}]_i$ were performed as previously reported [11,15]. The concentrations of the inhibitors used in this study were individually optimized with regard to Ca^{2+} release from internal stores for each cell type. The procedure for monitoring Mn^{2+} influx in hepatocytes and JURKAT cells was as described in [13] and [15] respectively, except that fura-2 fluorescence emission was recorded with a Sigma-ZWS II dual-wavelength spectrofluorimeter equipped with a xenon lamp. The fura-2 fluorescence signal from excitation at 334 and 360 nm was recorded separately to distinguish between alterations in $[\text{Ca}^{2+}]_i$ and Mn^{2+} -induced quenching of the dye. tBuBHQ and thapsigargin were dissolved in dimethyl sulphoxide; the final concentration of solvent in the cell incubation mixture never exceeded 0.5 % (v/v). Vasopressin was dissolved in water, and OKT3 in cell-suspension buffer.

RESULTS

Effects of thapsigargin, tBuBHQ and vasopressin on Ca^{2+} signalling in hepatocytes

Addition of tBuBHQ (20 μM) or thapsigargin (1 μM) to fura-2-loaded isolated hepatocytes, maintained in the presence of 1.3 mM extracellular Ca^{2+} , produced a rapid increase in $[\text{Ca}^{2+}]_i$ to a plateau (Figs. 1a and 1b) that was sustained for over 10 min. For both compounds the rise in $[\text{Ca}^{2+}]_i$ was concentration-dependent. Half-maximal and maximal $[\text{Ca}^{2+}]_i$ responses were

Abbreviations used: tBuBHQ, 2,5-di-(*t*-butyl)-1,4-benzohydroquinone; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration.

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Fig. 1. Effects of thapsigargin, tBuBHQ and vasopressin on $[Ca^{2+}]_i$ of hepatocytes in the absence or presence of extracellular Ca^{2+}

The $[Ca^{2+}]_i$ was measured by using the ratio of fura-2 fluorescence (500 nm emission) with excitation at 334 and 366 nm. Fura-2-loaded hepatocytes (10^6 cells/ml) were suspended in 1.3 mM- Ca^{2+} -containing (a, b) or nominally Ca^{2+} -free (c-e) medium; in the latter case, hepatocytes maintained in buffer containing 1.3 mM- Ca^{2+} were washed and resuspended in nominally Ca^{2+} -free medium (without EGTA) 90–120 s before addition of test compounds. The following additions were made as indicated by arrows: 20 μ M-tBuBHQ, 1 μ M-thapsigargin (TSG) and 10 nM-vasopressin (VP). Each trace is representative of at least four separate experiments.

Fig. 2. Effects of thapsigargin, tBuBHQ and OKT3 on $[Ca^{2+}]_i$ of JURKAT cells in the absence or presence of extracellular Ca^{2+}

The $[Ca^{2+}]_i$ was measured as described in the legend of Fig. 1. Fura-2-loaded JURKAT cells (1.5×10^6 cells/ml) were suspended in 1.3 mM- Ca^{2+} -containing (a-d, upper traces) or Ca^{2+} -free (1.3 mM- Ca^{2+} plus 3 mM-EGTA) (a-d lower traces, e and f) medium. The following additions were made as indicated by arrows: 20 μ M-tBuBHQ, 0.1 μ M-thapsigargin (TSG) and OKT3 (1:200 dilution), an antibody directed to the CD3 complex of the T-cell-receptor complex. Each trace is representative of at least four separate experiments.

obtained with 50 nM- and 200 nM-thapsigargin respectively (results not shown), whereas for tBuBHQ the half-maximal effect occurred with 2 μ M and maximal effect with 10 μ M (results not shown; [11]). Addition of thapsigargin (1 μ M) during the sustained phase induced by tBuBHQ (20 μ M) or vice versa, failed to produce any further change in $[Ca^{2+}]_i$ (Figs. 1a and 1b). Thus tBuBHQ and thapsigargin appeared to release Ca^{2+} from the same intracellular Ca^{2+} pool in hepatocytes. The addition of vasopressin (10 nM) after thapsigargin, tBuBHQ or a combination of both inhibitors produced no further increase in $[Ca^{2+}]_i$

(Figs. 1a and 1b), indicating that complete mobilization of the vasopressin-sensitive pool by these agents had occurred. In contrast, the hormone stimulated a rapid $[Ca^{2+}]_i$ decrease to near-basal level, in agreement with previous reports [11,16]. However, when vasopressin (10 nM) was added after a sub-maximal concentration of thapsigargin (e.g. 2 nM), a further rapid rise in $[Ca^{2+}]_i$ was observed, consistent with a partial release of the hormone-sensitive Ca^{2+} store by thapsigargin (results not shown).

In nominally Ca^{2+} -free medium (without added Ca^{2+} or

Fig. 3. Effects of thapsigargin, tBuBHQ and Ca^{2+} -mobilizing agonists on fluorescence of fura-2-loaded hepatocytes and JURKAT cells in the presence of MnCl_2

Fura-2 fluorescence (500 nm emission, expressed as arbitrary units) was measured with excitation at 360 nm (the isoemissive wavelength for fura-2), as outlined in the Materials and methods section. Activation of bivalent-cation entry results in quenching of intracellular fura-2 fluorescence. Cells were suspended in the presence of 1.3 mM- Ca^{2+} and MnCl_2 (a, 0.5 mM for hepatocytes; b, 50 μM for JURKAT cells). The following additions were made as indicated by arrows: 20 μM -tBuBHQ, 1 μM -thapsigargin (TSG) and 10 nM-vasopressin (VP) to hepatocytes (a), or 20 μM -tBuBHQ, 0.1 μM -thapsigargin (TSG), OKT3 (1:200 dilution) and carrier solvent to JURKAT cells (b). The thapsigargin and tBuBHQ traces are superimposed in (a), since the results obtained were identical. Each trace is representative of at least four separate experiments.

EGTA), thapsigargin (1 μM) or tBuBHQ (20 μM) caused a transient rather than a sustained rise in $[\text{Ca}^{2+}]_i$. Addition of tBuBHQ after thapsigargin, or vice versa, failed to produce a further release of internal Ca^{2+} (Figs. 1c and 1d). The very rapid increase in $[\text{Ca}^{2+}]_i$ after addition of vasopressin in Ca^{2+} -free medium (Fig. 1e) was followed by a decrease at a rate that was faster than that produced by thapsigargin or tBuBHQ. When thapsigargin was added after vasopressin (10 nM), only a very small transient elevation of $[\text{Ca}^{2+}]_i$ was observed (Fig. 1e), followed by a decrease in $[\text{Ca}^{2+}]_i$ to a steady state lower than that reached by the hormone alone, owing to the combination of two effects: inhibition of the Ca^{2+} -reuptake system by thapsigargin and stimulation of Ca^{2+} efflux by vasopressin. Similar results were obtained with tBuBHQ (results not shown; [11]). These results indicate that in intact hepatocytes thapsigargin and tBuBHQ release a common Ca^{2+} store that is identical with, or marginally larger than, that mobilized by vasopressin.

Effects of thapsigargin, tBuBHQ and OKT3 on Ca^{2+} signalling in JURKAT cells

Addition of thapsigargin (100 nM) to fura-2-loaded JURKAT cells, maintained in the presence of 1.3 mM extracellular Ca^{2+} , evoked a two-phase increase in $[\text{Ca}^{2+}]_i$ (Fig. 2a, upper trace). An initial rapid increase was observed, followed by a slower rise reaching a plateau, that was sustained for over 10 min (results not shown). Increasing thapsigargin concentration to 1 μM did not increase either phase of $[\text{Ca}^{2+}]_i$ elevation any further. Similar changes in $[\text{Ca}^{2+}]_i$ were observed when tBuBHQ (20 μM) was added to JURKAT cells (Fig. 2b, upper trace). However, lower concentrations of thapsigargin (10 nM) or tBuBHQ (1 μM), which result in partial inhibition of the microsomal Ca^{2+} -ATPase, produced a slower release of internal Ca^{2+} stores, resulting in a monophasic rise in $[\text{Ca}^{2+}]_i$ (results not shown). The magnitude of the peak increase in $[\text{Ca}^{2+}]_i$ elicited by OKT3 (Figs. 2c and 2d, upper traces), an antibody directed to CD3 in the T-cell-receptor complex [17], was at least twice that of the initial phase of $[\text{Ca}^{2+}]_i$ increase caused by either thapsigargin or tBuBHQ.

In Ca^{2+} -free medium (in the presence of 1.3 mM- Ca^{2+} and 3 mM-EGTA), both thapsigargin and tBuBHQ caused a Ca^{2+} transient in JURKAT cells (Figs. 2a and 2b, lower traces). The second, sustained, phase of the increase in $[\text{Ca}^{2+}]_i$ after thapsigargin or tBuBHQ stimulation was abolished in the absence of extracellular Ca^{2+} , suggesting the involvement of Ca^{2+} entry, whereas the first phase was virtually identical, which is consistent with the mobilization of intracellular Ca^{2+} stores. In the absence of extracellular Ca^{2+} , the peak increase in Ca^{2+} evoked by OKT3 in JURKAT cells was lower and the sustained phase was abolished (Figs. 2c and 2d, lower traces). This suggests that the initial transient increase in $[\text{Ca}^{2+}]_i$ by OKT3 consists of Ca^{2+} influx from the extracellular medium, in addition to Ca^{2+} mobilization from internal stores, whereas the sustained phase is dependent on Ca^{2+} entry. In the presence of EGTA, the time taken for the $[\text{Ca}^{2+}]_i$ to reach a peak after OKT3 stimulation was short, as compared with that with either thapsigargin or tBuBHQ. This indicates that the rate of emptying the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} pool by $\text{Ins}(1,4,5)\text{P}_3$ after OKT3 stimulation is much faster than the rate of Ca^{2+} release from this store owing to inhibition of the microsomal Ca^{2+} translocase.

Addition of OKT3 (1:200 dilution) to JURKAT cells after thapsigargin- or tBuBHQ-induced Ca^{2+} transients (in Ca^{2+} -free medium) did not produce any further increase in $[\text{Ca}^{2+}]_i$ (Figs. 2a and 2b, lower traces). Under similar conditions, addition of thapsigargin (100 nM) or tBuBHQ (20 μM) to JURKAT cells after the OKT3-induced Ca^{2+} transient had returned to basal level caused a very small increase in $[\text{Ca}^{2+}]_i$ (Figs. 2c and 2d, lower traces). Addition of thapsigargin to JURKAT cells after the tBuBHQ-induced increase in $[\text{Ca}^{2+}]_i$ had returned to basal level, and vice versa, did not further increase $[\text{Ca}^{2+}]_i$ (Figs. 2e and 2f). Taken together, these data suggest that both thapsigargin and tBuBHQ mobilize a common pool that is identical with, or just marginally larger than, the OKT3-sensitive Ca^{2+} pool in JURKAT cells.

Comparison of the effects of thapsigargin, tBuBHQ and Ca^{2+} -mobilizing agonists on Mn^{2+} entry in hepatocytes and JURKAT cells

The role of the state of the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} pool in regulating Ca^{2+} influx has recently gained support from studies with thapsigargin [5,7]. In the present work we have compared the effects of thapsigargin and tBuBHQ, and those of Ca^{2+} -mobilizing agonists, on Ca^{2+} entry into hepatocytes and JURKAT cells, using a Mn^{2+} -influx technique [18]. Different Mn^{2+} concentrations and time elapsed between addition of test compounds and Mn^{2+} were tested, and the conditions used in Fig. 3 are those that produced optimal sensitivity. The concentration of Mn^{2+} used in hepatocytes was higher than that in JURKAT cells, owing to the lower basal permeability to Mn^{2+} in hepatocytes.

Fig. 3(a) shows that, in the presence of 1.3 mM extracellular Ca^{2+} , the addition of vasopressin to hepatocytes stimulated Mn^{2+} quenching of fura-2 fluorescence only after a lag period of about 20–30 s (see also [13]). However, thapsigargin and tBuBHQ completely failed to stimulate this entry route in hepatocytes under the conditions employed. Similar results were obtained when hepatocytes were resuspended in nominally Ca^{2+} -free medium, although the basal and stimulated rates were faster (results not shown).

Similar experiments on the effects of thapsigargin and tBuBHQ on Mn^{2+} entry were performed in JURKAT cells. Fig. 3(b) shows that upon addition of thapsigargin (100 nM) or tBuBHQ (20 μM) there was a lag phase of about 2 min before any apparent decrease in fura-2 fluorescence owing to Mn^{2+} was observed. This latency supports the idea that the second phase of increase

in $[Ca^{2+}]_i$ (Figs. 2a and 2b) is due to Ca^{2+} entry. In contrast, quenching of fura-2 fluorescence was noticeable within 30–60 s of OKT3 (1:200 dilution) stimulation; however, the rates of bivalent-cation entry were similar for all three compounds, once it occurred. These results indicate that emptying the $Ins(1,4,5)P_3$ -sensitive Ca^{2+} pool stimulates Ca^{2+} uptake in JURKAT cells, which is consistent with previous findings [19]. These observations also suggest that the $Ins(1,4,5)P_3$ -sensitive Ca^{2+} pool may have to be emptied to a certain extent before Ca^{2+} entry is triggered.

DISCUSSION

The present results show that thapsigargin and tBuBHQ mobilize a common intracellular Ca^{2+} store in isolated hepatocytes as well as in JURKAT cells. The slight rise in $[Ca^{2+}]_i$ observed after addition of thapsigargin or tBuBHQ (cf. Figs. 1e and 2c–2d) may indicate that these agents deplete a pool which is marginally larger than the agonist-releasable one. Another interpretation of this effect is that, in the presence of the agonist, Ca^{2+} cycling may occur across the membrane of the intracellular Ca^{2+} store, i.e. part of the Ca^{2+} released into the cytosol through $Ins(1,4,5)P_3$ -gated channels may be taken up into the store by the Ca^{2+} -ATPase. Subsequent addition of tBuBHQ or thapsigargin would inhibit the sequestration mechanism, and therefore further increase $[Ca^{2+}]_i$. Alternatively, the levels of Ca^{2+} within the $Ins(1,4,5)P_3$ -sensitive store may modulate the release of Ca^{2+} by $Ins(1,4,5)P_3$ [20]. This implies that there would be a level of intraluminal $[Ca^{2+}]$ at which $Ins(1,4,5)P_3$ would not release any more Ca^{2+} ; however, the inhibitors of the microsomal Ca^{2+} -ATPase would still be able to mobilize the remaining Ca^{2+} from the stores. Furthermore, it has been shown that raising $[Ca^{2+}]_i$ in the cytosol can inhibit Ca^{2+} release by $Ins(1,4,5)P_3$ [21], leading to a partial refilling of the intracellular stores.

In many non-excitabile cells, Mn^{2+} enters the cell through receptor-/second-messenger-operated Ca^{2+} channels [18]. We have previously reported that tBuBHQ, in contrast with Ca^{2+} -mobilizing hormones, does not stimulate Mn^{2+} entry into hepatocytes [13]. However, pretreatment with tBuBHQ markedly enhanced the rate of vasopressin-induced Mn^{2+} influx, suggesting that emptying of intracellular Ca^{2+} pool(s), although not sufficient for activation, might control the rate of influx through this agonist-stimulated bivalent-cation entry pathway. In the present work, we have extended these observations to thapsigargin, and found that neither inhibitor stimulated Ca^{2+} entry into hepatocytes, as assessed by the Mn^{2+} -influx technique (cf. Fig. 3a), although pretreatment with either of them caused a very marked stimulation of the rate of hormone-induced Mn^{2+} influx ([13]; J. Llopis, G. E. N. Kass, A. Gahm & S. Orrenius, unpublished work). The observation that thapsigargin or tBuBHQ produced a sustained elevation of $[Ca^{2+}]_i$ in hepatocytes in the presence of extracellular Ca^{2+} (Figs. 1a and 1b) but a transient rise in its absence (cf. Figs. 1c and 1d) may indicate that a Ca^{2+} -entry component is maintaining the sustained plateau in the former case (as concluded in [1]). However, removal of extracellular Ca^{2+} would favour net extrusion of the Ca^{2+} released by thapsigargin or tBuBHQ, leading to a decrease in $[Ca^{2+}]_i$. In addition, it is also possible that these inhibitors stimulate Ca^{2+} entry through a pathway that does not admit Mn^{2+} . Indeed, Sage *et al.* [22] proposed the existence of at least three different receptor-mediated Ca^{2+} -entry mechanisms in human platelets, with different selectivity for Mn^{2+} . This may also apply to hepatocytes, which would explain the lack of stimulation of Mn^{2+} entry by thapsigargin or tBuBHQ.

In JURKAT cells, however, discharge of internal Ca^{2+} stores

by either thapsigargin or tBuBHQ activated Mn^{2+} entry (cf. Fig. 3b), with a rate similar to that produced by the agonist OKT3. This is in agreement with a recent report that emptying the $Ins(1,4,5)P_3$ -sensitive Ca^{2+} pool in JURKAT cells independently of $Ins(1,4,5)P_3$ production, by using non-esterified fatty acids, stimulates the influx of Ca^{2+} across the plasma membrane [19].

In conclusion, both tBuBHQ and thapsigargin, which have been shown to inhibit selectively the liver microsomal Ca^{2+} -ATPase activity [1,10], produced virtually identical results with respect to Ca^{2+} mobilization from internal stores and activation of bivalent-cation entry across the plasma membrane. However, differences were found in the stimulation of Mn^{2+} entry by either of the inhibitors between hepatocytes and JURKAT cells. Therefore, the contrasting results reported [11,13,23,24] regarding Mn^{2+} entry by tBuBHQ and thapsigargin most likely reflect different properties of the bivalent-cation-influx pathway(s) between cell types, rather than dissimilarities in the mechanism of action of the two inhibitors.

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