Inhibition of L-type calcium-channel activity by thapsigargin and 2,5-t-butylhydroquinone, but not by cyclopiazonic acid

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Thapsigargin (TG), 2,5-t-butylhydroquinone (tBHQ) and cyclopiazonic acid (CPA) all inhibit the initial Ca^{2+}-response to thyrotropin-releasing hormone (TRH) by depleting intracellular Ca^{2+} pools sensitive to inositol 1,4,5-trisphosphate (IP_3). Treatment of GH_3 pituitary cells for 30 min with 5 nM TG, 500 nM tBHQ or 50 nM CPA completely eliminated the TRH-induced spike in intracellular free Ca^{2+} ($[Ca^{2+}]_i$). Higher concentrations of TG and tBHQ, but not CPA, were also found to inhibit strongly the activity of L-type calcium channels, as measured by the increase in $[Ca^{2+}]_i$ or $^{45}Ca^{2+}$ influx stimulated by depolarization. TG and tBHQ blocked high-K^+-stimulated $^{45}Ca^{2+}$ uptake, with IC_{50} values of 10 and 1 μM respectively. Maximal inhibition of L-channel activity was achieved 15–30 min after drug addition. Inhibition by tBHQ was reversible, whereas inhibition by TG was not. TG and CPA did not affect spontaneous $[Ca^{2+}]_i$ oscillations when tested at concentrations adequate to deplete the IP_3-sensitive Ca^{2+} pool. However, 20 μM TG and 10 μM tBHQ blocked $[Ca^{2+}]_i$ oscillations completely. The effect of drugs on calcium currents was measured directly by using the patch-clamp technique. When added to the external bath, 10 μM CPA caused a sustained increase in the calcium-channel current amplitude over 8 min, 10 μM tBHQ caused a progressive inhibition, and 10 μM TG caused an enhancement followed by a sustained block of the calcium current over 8 min. In summary, CPA depletes IP_3-sensitive Ca^{2+} stores and does not inhibit voltage-operated calcium channels. At sufficiently low concentrations, TG depletes IP_3-sensitive stores without inhibiting L-channel activity, but, for tBHQ, inhibition of calcium channels occurs at concentrations close to those needed to block agonist mobilization of intracellular Ca^{2+}.

INTRODUCTION

Several signal-transduction pathways lead to the activation of phospholipase C, which increases the rate of hydrolysis of phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate (IP_3) [1,2]. IP_3, acting at an intracellular receptor on the endoplasmic or sarcoplasmic reticulum [3], releases Ca^{2+} from intracellular stores with an intraluminal Ca^{2+} concentration greater than that in the cytoplasm. The gradient between Ca^{2+} concentrations in the cytoplasm and extracellular fluid or between the cytoplasm and the endoplasmic or sarcoplasmic reticulum is established in part by Ca^{2+}-dependent ATPases [4–7]. A number of genes encoding sarcosplamic- or endoplasmic-reticulum calcium (SERCA) ATPases have been cloned. The SERCA ATPases are structurally similar enzymes differing primarily in their C-terminal sequences. SERCA ATPases can be distinguished from plasma-membrane calcium (PMCA) ATPases on the basis of their structure and their sensitivity to a number of drugs [5–7]. Thapsigargin (TG), a sesquiterpene lactone isolated from plants, forms an essentially irreversible complex with all known SERCA ATPases at stoichiometric concentrations, but TG does not inhibit PMCA or Na^+/K^+-ATPases [6,8–11]. 2,5-t-Butylhydroquinone (tBHQ) is another selective SERCA ATPase inhibitor that is less potent and more reversible than TG [12–15]. A mycotoxin, cyclopiazonic acid (CPA), is a third member of this class [16,17]. By inhibiting the SERCA ATPases, TG, tBHQ and CPA all deplete sarcoplasmic- or endoplasmic-reticulum Ca^{2+} stores, presumably because of an endogenous Ca^{2+} leak. As a result, all three drugs can prevent agonists that increase IP_3 from generating a Ca^{2+} signal without interfering with IP_3 production [2,5,18]. These inhibitors are also useful because they generate a transient rise in intracellular Ca^{2+} and mimic the actions of Ca^{2+}-mobilizing hormones.

TG, tBHQ and CPA are selective in inhibiting SERCA ATPases, since none of them has an effect on plasma-membrane Ca^{2+} pumps. There are reported differences in the efficacies of the inhibitors, depending on cell type, and they are not absolutely specific, showing some apparent sites of action besides the SERCA ATPases. Both tBHQ and CPA have been reported to block uptake of extracellular Ca^{2+} that often occurs in response to depletion of intracellular stores [9,20], an action not shared by TG. CPA has been reported to activate an influx pathway for bivalent cations [18].

We have studied the effects of TG, tBHQ and CPA in the GH_3 line of rat pituitary tumour cells in culture. GH_3 cells respond to a prototypical Ca^{2+}-mobilizing agonist, thyrotropin-releasing hormone (TRH), with a biphasic increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) [21]. The initial $[Ca^{2+}]_i$ spike is independent of extracellular Ca^{2+} and is believed to be due to the release of intracellular Ca^{2+} stimulated by IP_3 [22,23]. GH_3 cells are electrically excitable, and most cells display spontaneous action potentials and calcium oscillations that depend on the activity of dihydropyridine-sensitive L-type Ca^{2+} channels [24–27]. We report here that TG, tBHQ and CPA all deplete IP_3-sensitive stores, but that TG and tBHQ also inhibit L-type Ca^{2+} channels. Inhibition of L-type channels occurs at TG concentrations above those required to block the $[Ca^{2+}]_i$ response to

Abbreviations used: tBHQ, 2,5-t-butylhydroquinone; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CPA, cyclopiazonic acid; IP_3, inositol 1,4,5-trisphosphate; SERCA ATPase, sarcoplasmic- or endoplasmic-reticulum Ca^{2+}-ATPases; TG, thapsigargin; TRH, thyrotropin-releasing hormone; HBSS, Hanks' balanced salt solution.

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TRH, but for tBHQ the concentrations are near those required to deplete the IP_3-sensitive Ca^{2+} pool. The inhibition appears to be a direct effect at the Ca^{2+} channel.

MATERIALS AND METHODS

Materials

$^{45}Ca^{2+}$ as $CaCl_2$ (17 mCi/mg) was obtained from New England Nuclear Corp. (Boston, MA, U.S.A.) or Amersham (Arlington Heights, IL, U.S.A.). tBHQ, Hepes and salts were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). CPA and TG were from Research Biochemicals (Natick, MA, U.S.A.) and tBHQ was from Aldrich (Milwaukee, WI, U.S.A.). Tissue-culture media and sera were from Grand Island Biological Co. (Grand Island, NY, U.S.A.) and tissue-culture plasticware was from Corning (Corning, NY, U.S.A.). BAY K8644 was a gift from Miles Institute of Preclinical Pharmacology (New Haven, CT, U.S.A.). Fura2/AM was from Molecular Probes (Eugene, OR, U.S.A.). Dyes and drugs were stored in dimethyl sulphoxide and diluted at least 100-fold into medium or buffer immediately before use.

Methods

Rat pituitary GH_3 cells were grown in monolayer culture in F10 medium supplemented with 15% horse serum and 2.5% fetal-calf serum [28]; this is referred to as complete F10 medium. Serum-free F10 medium contains 126 mM NaCl, 3.8 mM KCl, 0.3 mM $CaCl_2$ and other ions as noted by the manufacturer. For experiments, GH_3 cells from a single donor culture were inoculated into 35 mm- or 100 mm-diam. dishes and grown for 4–8 days before use. Fresh medium was added 24–48 h before experiments. $^{45}Ca^{2+}$ uptake was measured as previously described [29]. Unless noted, 35 mm dishes of cells were incubated for 10 min at 37 °C in serum-free F10 medium containing 1 μCi/ml $^{45}Ca^{2+}$. Depolarization-stimulated uptake was defined as the difference between uptake in medium alone and uptake in medium containing 50 mM KCl + 1 μM BAY K8644. $^{45}Ca^{2+}$ uptake was typically stimulated 5–20-fold by depolarization in the presence of the Ca^{2+}-channel agonist. Data shown are means and range or S.E.M. of 2–3 dishes. Experiments were performed on at least two occasions with similar results.

$[Ca^{2+}]_i$ was measured in cells suspended in Hanks' balanced salt solution supplemented with 15 mM Hepes at pH 7.4 (HBSS). Cells were loaded for 30 min at 37 °C with 2 μM fura-2/AM by published methods [30] and, where noted, drugs were included in the loading period and during fluorimetry. Cells at a density of $(2–5) \times 10^6$ cells/ml were placed in a cuvette thermostatically maintained at 37 °C and kept in suspension with a magnetic stir-bar.

Ca^{2+}-channel currents were measured in isolated cells with the whole-cell arrangement of the patch-clamp technique [31]. Electrodes (resistance of 3–5 MΩ) were fabricated from Gold Seal Accu-fill 90 Micopets (Clay Adams, Parsippany, NJ, U.S.A.). Currents were recorded at room temperature (22–25 °C) with a Yale Mark IV patch-clamp amplifier and headstage. Series resistance compensation was used in all experiments, and was adjusted to give the fastest possible transients without producing ringing. Data were sampled at 150 μs and filtered at 2 kHz with a low-pass 8-pole Bessel filter, digitized, and then stored on a Dell PC-386 computer. Current–voltage plots were generated by applying 100 ms voltage pulses at 1 Hz to various test potentials from a holding potential of −40 mV, chosen to eliminate Na^+ currents and T-type Ca^{2+}-channel currents.

Cells were bathed in normal Tyrode solution containing (in mM): NaCl 132, KCl 4.8, $MgCl_2$ 3, $CaCl_2$ 1, dextrose 5 and Hepes 10, pH 7.4. After establishment of whole-cell voltage-clamp, external solution was changed to one containing (in mM): *N*-methyl-D-glucamine 125, CsCl 5, $BaCl_2$ 40, Hepes 10, $MgCl_2$ 1 and dextrose 5, pH 7.4. The standard pipette solution contained (in mM): CsCl 60, $CaCl_2$ 1, Hepes 10, $MgCl_2$ 1, EGTA 11, K_2ATP 10, aspartic acid 50, pH 7.4. Drugs at the appropriate concentrations were included in the external bath solution. Solutions were changed by using a multibarrelled ejection pipette, which allows for fast local concentration changes. During each experiment, cells were continuously perfused with drug-free solution or solution containing an appropriate concentration of drug. Differences between groups were determined by Student's *t* test.

Intracellular imaging of cells loaded with fura-2 was performed as follows. GH_3 cells grown on glass coverslips coated with Cell-Tak from Collaborative Biomedical Products (Bedford, MA, U.S.A.) were washed and loaded with 2–4 μM fura-2/AM, 0.1% BSA and 5 μg/ml cyclosporin A in HBSS for 30 min at room temperature; loading buffer included drugs where noted. The coverslips were rinsed three times in buffer and placed in a Sykes–Moore chamber (Bellco, Vineland, NJ, U.S.A.) maintained at 25 °C with a Fryer heating stage (Frank E. Fryer Co., Carpentersville, IL, U.S.A.) and left to equilibrate for 20 min before the experiment. TRH or KCl was added as 250 μl of a 4-fold-concentrated stock solution in buffer to 750 μl of buffer in the cell chamber to ensure rapid mixing. Control addition of 250 μl of buffer alone did not affect fura-2 fluorescence or disrupt the image. Images were routinely captured on a Nikon inverted microscope fitted with 340 nm and 380 nm filters designed for fura-2 ratio imaging (Omega Optical, Brattleboro, VT, U.S.A.), using a Dage CCD 72 camera and Geniisys image-intensifier system (Dage-MTI, Michigan City, IN, U.S.A.) and Image I/AT image processing and fura-2 analysis software from Universal Imaging (Media, PA, U.S.A.). Ratio images were normally captured and analysed every 1200 ms after excitation at 340 nm and 380 nm by using a Ludl 6 filter and shutter wheel (Ludl Electronic Products, Hawthorne, NY, U.S.A.) with 0.8 neutral-density filter. In a few experiments designed to optimize temporal resolution, images were captured every 500 ms at 380 nm only. A $[Ca^{2+}]_i$ calibration *in vivo* [32] was performed at the end of each experiment at the same camera gain settings, as follows. The GH_3 cells were rinsed three times with Ca^{2+}-free HBSS with 15 mM Hepes at pH 7.15. The following additions were made to the cells in Ca^{2+}-free HBSS to equilibrate the Ca^{2+}: 3 mM bis-(*o*-aminophenoxy)ethane-*NNN'N'*-tetra-acetic acid (BAPTA) to chelate any residual extracellular calcium, 20 μM tetrakis-(2-pyridylmethyl)ethylenediamine to chelate intra- and extracellular heavy metals, 15 μM monensin and 15 μM nigericin to dissipate cellular ion gradients, and 10 μM TG to prevent re-uptake into TG-sensitive intracellular Ca^{2+} pools. 4-Bromo A23187 (5 μM) was added 10–20 min later to equilibrate intracellular Ca^{2+} with extracellular Ca^{2+}. After 30 min the minimum fura-2 fluorescence at 340 nm and 380 nm at $[Ca^{2+}]_i = 0$ was recorded for 10 cells. The fluorescence of these same 10 cells was again recorded after adding 8–10 mM $CaCl_2$ to find the maximum fura-2 fluorescence at 340 nm and 380 nm. $[Ca^{2+}]_i$ was calculated from the relationship $[Ca^{2+}]_i = K_d \times (F_{min.}/F_{max.}) \times [(R - R_{min})/(R_{max.} - R)]$, where $K_d = 257$ nM at 25 °C, $R_{min.}$ and $R_{max.}$ were the ratios recorded at minimum and maximum $[Ca^{2+}]_i$, $F_{min.}$ and $F_{max.}$ were fluorescence values determined at 380 nm with the minimum and maximum $[Ca^{2+}]_i$, and R the experimental 340 nm/380 nm fluorescence ratio [32].

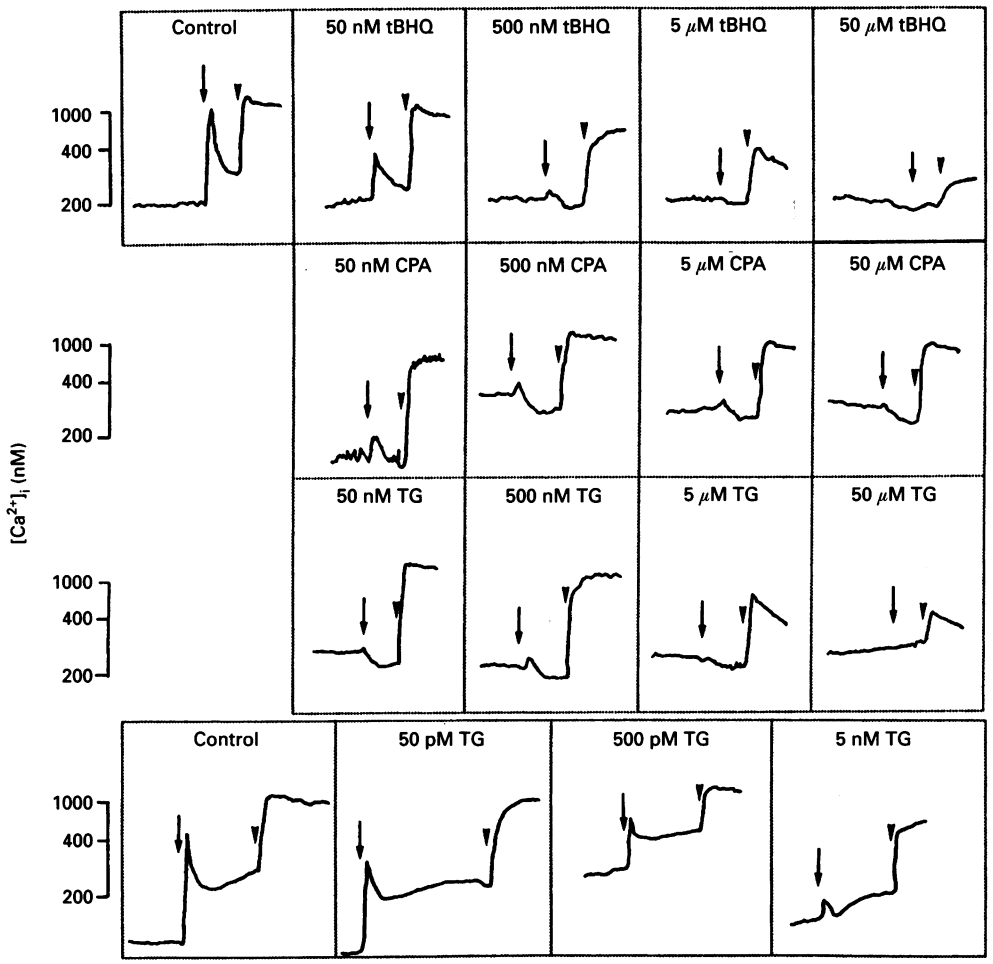

Figure 1 Effect of TG, tBHQ and CPA on TRH- and K^+-stimulated $[Ca^{2+}]_i$ responses

Cells were loaded with fura-2 for 30 min in the presence of tBHQ, CPA or TG at the concentrations shown. Cells were pelleted and resuspended in HBSS containing the same additions. At the time shown by the left arrow of each panel, 100 nM TRH was added. Either 1 min (top three rows) or 2 min (bottom row) later, 50 mM KCl was added, as noted by the arrowhead on the right. The scale on the left is the calculated $[Ca^{2+}]_i$.

RESULTS

Treatment of GH_3 cells with TG, tBHQ or CPA completely prevented the initial increase in $[Ca^{2+}]_i$ generated by TRH. In the experiment shown in Figure 1, cells were incubated with different concentrations of drugs during the 30 min loading with fura-2/AM and during the measurement of $[Ca^{2+}]_i$. The initial Ca^{2+} spike evoked by TRH was completely blocked by 5 nM TG, 500 nM tBHQ and 500 nM CPA. In this protocol, half-maximal inhibition of the TRH response required < 50 nM CPA and ~ 50 nM tBHQ and 0.5 nM TG. When the $[Ca^{2+}]_i$ spike was eliminated by depleting the IP_3-sensitive Ca^{2+} stores with drugs, TRH actually decreased $[Ca^{2+}]_i$ by stimulating Ca^{2+} efflux from the cytoplasm.

In GH_3 cells, the Ca^{2+} response to depolarization, measured as the depolarization-stimulated increase in either $[Ca^{2+}]_i$ or $^{45}Ca^{2+}$ influx, is almost exclusively the result of the influx through dihydropyridine-sensitive L-type Ca^{2+} channels. The Ca^{2+} response to depolarization can be inhibited by Ca^{2+}-channel blockers such as nimodipine or amplified by Ca^{2+}-channel agonists such as BAY K8644. The effect of the SERCA ATPase inhibitors on the depolarization-stimulated increase in $[Ca^{2+}]_i$ was determined by challenging cells treated with TG, tBHQ or CPA with high K^+ after TRH (Figure 1). $[Ca^{2+}]_i$ typically increased to 1–2 μM when GH_3 cells were depolarized with 50 mM K^+. CPA did not affect the $[Ca^{2+}]_i$ response to high K^+. However, TG and tBHQ did blunt the depolarization response at high concentrations. At 10 μM or above, TG and tBHQ decreased the $[Ca^{2+}]_i$ response to high K^+ to 400 nM or less. The peak $[Ca^{2+}]_i$ response to depolarization was decreased by half at 0.5 μM tBHQ and 5 μM TG.

$^{45}Ca^{2+}$ influx stimulated by high K^+ and BAY K8644 was monitored in the presence of different concentrations of TG, tBHQ and CPA (Figure 2). TG and tBHQ nearly completely blocked stimulated $^{45}Ca^{2+}$ uptake, with IC_{50} values of 10 and 1 μM respectively. In contrast, CPA slightly increased $^{45}Ca^{2+}$ uptake at sub-micromolar doses and was not inhibitory at doses up to 100 μM. The effects of TG and tBHQ were observed at added KCl concentrations of 6–50 mM with or without the L-channel agonist BAY K8644 (Figure 3). BAY K8644 at concentrations from 0.25 to 2.5 μM failed to reverse the inhibition caused by TG and tBHQ (results not shown).

To measure the rate at which the drugs inhibited Ca^{2+}-channel activity, cultures were incubated with 10 μM TG or 2 μM tBHQ

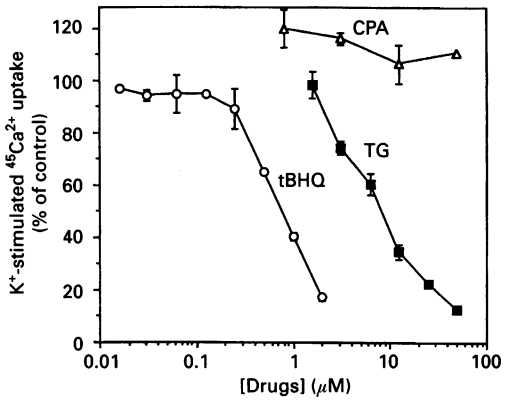

Figure 2 Effect of drug concentration on depolarization-stimulated $^{45}Ca^{2+}$ uptake

Cells were incubated for 30 min with tBHQ (○), TG (■) or CPA (△) at the concentrations shown. $^{45}Ca^{2+}$ (1 μCi/ml) was then added with no additions or 50 mM KCl and 1 μM BAY K8644. Data shown are depolarization-stimulated $^{45}Ca^{2+}$ uptake, calculated as the difference in uptake with and without added KCl and BAY K8644.

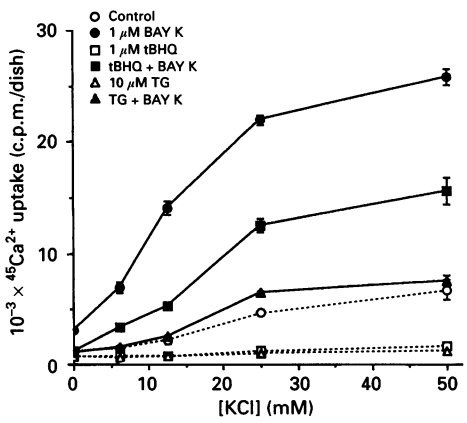

Figure 3 Effects of depolarization and BAY K8644 on drug inhibition of $^{45}Ca^{2+}$ uptake

Cells were incubated in medium containing no drug (○, ●), 1 μM tBHQ (□, ■), or 10 μM TG (△, ▲). $^{45}Ca^{2+}$ was added with KCl at the concentrations shown for a 10 min incubation with no further additions (○, □, △) or 1 μM BAY K8644 (●, ■, ▲).

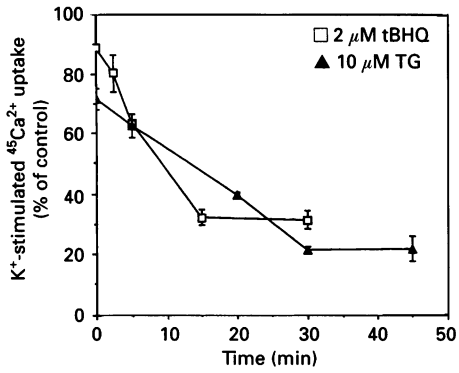

Figure 4 Rate of drug inhibition of L-channel activity

Cells were incubated for the times shown with 2 μM tBHQ (□) or 10 μM TG (▲). During the last 2.5 min (tBHQ experiment) or 5 min (TG experiment), 1 μCi/ml $^{45}Ca^{2+}$, 1 μM BAY K8644 and 50 mM KCl were added for the measurement of depolarization-stimulated $^{45}Ca^{2+}$ uptake. Data are expressed as means ± S.E.M. ($n = 3$) relative to control without drug.

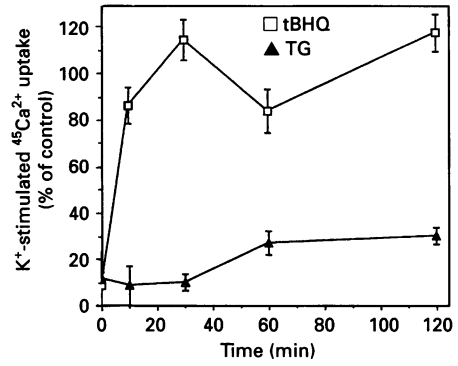

Figure 5 Rate of reversal of drug inhibition of L-channel activity

Cells were incubated for 30 min with 2 μM tBHQ (□) or 10 μM TG (▲). Medium was removed, and the cells were washed once and incubated in medium without drugs. At intervals, depolarization-stimulated $^{45}Ca^{2+}$ uptake was measured. Control dishes not exposed to drugs were included at each time point. Data for drug-treated cultures are expressed as a percentage of control.

for different periods before depolarization-stimulated $^{45}Ca^{2+}$ uptake was measured (Figure 4). Inhibition increased to a maximum over about 15 min for tBHQ and slightly more slowly, over 30 min, for TG. The rate of reversal of the inhibition was measured by washing the drugs out and monitoring Ca^{2+}-channel activity at intervals (Figure 5). Inhibition due to tBHQ was largely reversible, with depolarization-stimulated $^{45}Ca^{2+}$ uptake nearly restored to control levels after 10 min. Inhibition due to TG was almost irreversible over 2 h. The depletion of intracellular Ca^{2+} pools was similarly more prolonged when caused by TG than by tBHQ. Cells that had been incubated for 30 min with 50 nM TG did not show a TRH-induced $[Ca^{2+}]_i$ spike 30 min after drug removal, whereas cells that had been incubated with 500 nM tBHQ displayed a partial TRH response 20 min after drug wash-out (results not shown).

GH_3 cells display spontaneous oscillations in $[Ca^{2+}]_i$ that depend on the activity of L-type Ca^{2+} channels [24–27]. The effects of tBHQ, CPA and TG on these spontaneous oscillations were measured in fura-2-loaded single cells in a microscope equipped for fluorescence ratio imaging. As illustrated in Figure 6, TRH stimulated a rapid $[Ca^{2+}]_i$ spike, which was typically followed by a period without oscillations. A typical cell incubated with 50 nM TG displayed no $[Ca^{2+}]_i$ spike, consistent with depletion of IP_3-sensitive pools, and the period of inactivity was still observed (Figure 6). Similar patterns were seen with 20 μM CPA (results not shown). In order to analyse $[Ca^{2+}]_i$ oscillations more carefully, we measured 380 nm fluorescence every 500 ms for control cells and cells treated with 50 nM TG. TG did not alter the frequency, amplitude or shape of spontaneous $[Ca^{2+}]_i$ oscillations, which occurred at ~ 7/min (Figure 6), nor did 20 μM CPA (results not shown), indicating that these oscillations do not depend on IP_3-sensitive intracellular Ca^{2+} pools. However, 10 μM tBHQ and 20 μM TG blocked $[Ca^{2+}]_i$ oscillations completely and inhibited the response to depolarization with high K^+ (Figure 7). CPA did not alter the $[Ca^{2+}]_i$ response to depolarization even at 20 μM, nor did TG at 50 nM (Figure 7).

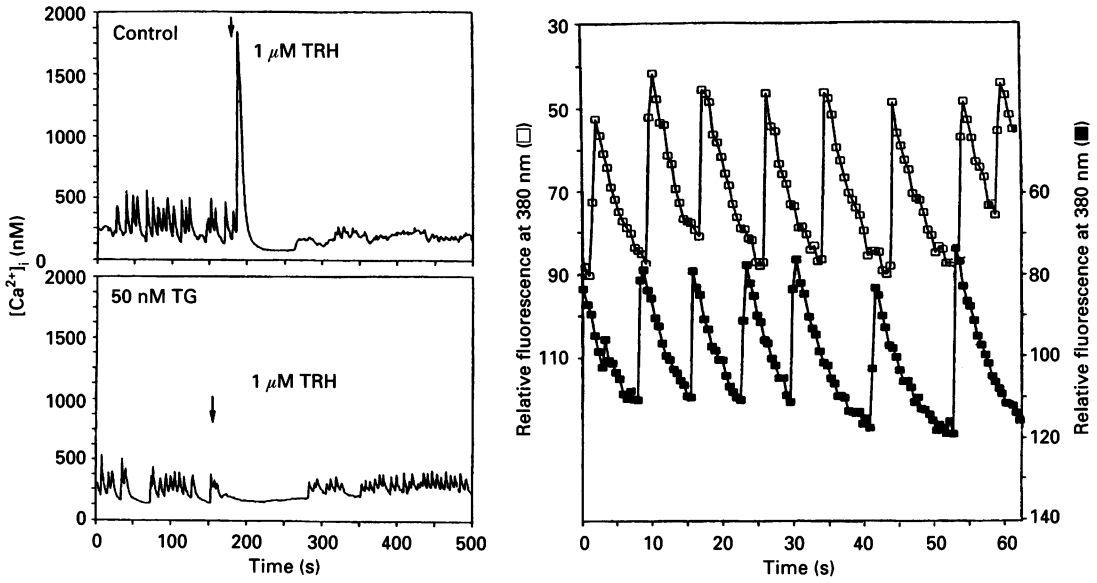

Figure 6 Effect of TG on spontaneous $[Ca^{2+}]_i$ oscillations and TRH responses measured in single cells by fluorescence ratio imaging

The left panels show the $[Ca^{2+}]_i$ of individual cells loaded with fura-2 without (upper panel) or with (lower panel) 50 nM TG during the 30 min loading and experimental period at 25 °C. Fluorescence emission was measured after excitation at 340 nm and 380 nm every 1200 ms, and the 340 nm/380 nm ratios were converted into $[Ca^{2+}]_i$ as described in the Materials and methods section. At the time shown by the arrow, TRH (1 μM final concn.) was added. The right panel shows spontaneous Ca^{2+} oscillations in control cells (■) and cells incubated with 50 nM TG (□). To increase the time resolution, florescence was measured every 500 ms at 380 nm only; relative fluorescence is shown.

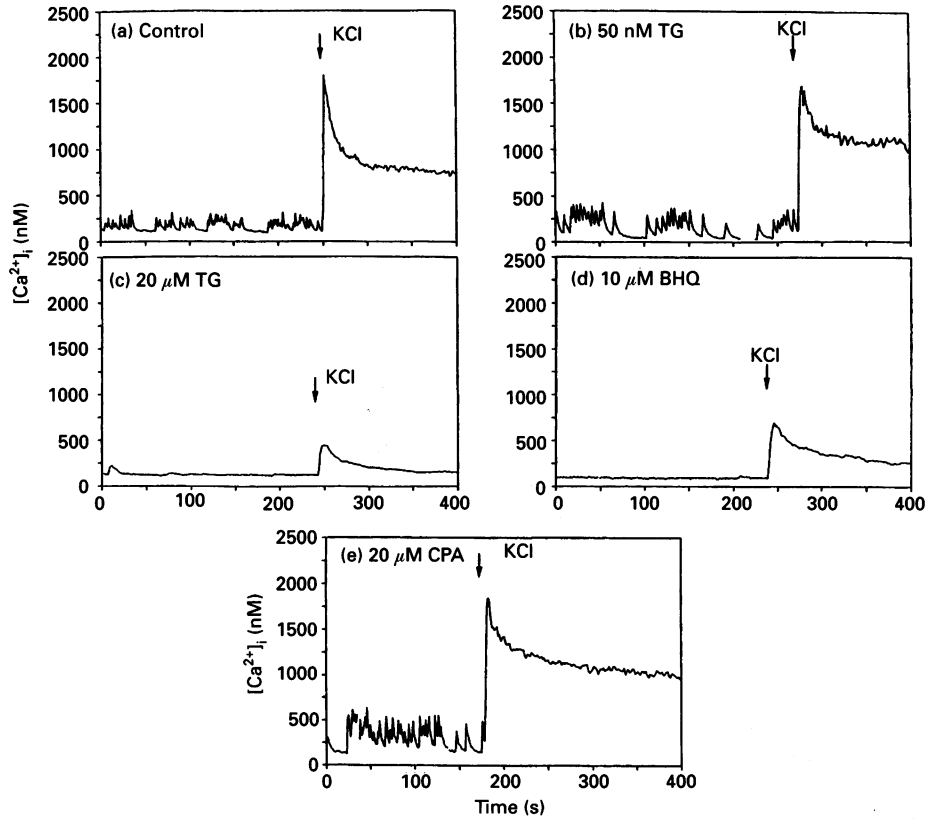

Figure 7 Effect of drugs on $[Ca^{2+}]_i$ oscillations and depolarization responses in single cells

Cells were loaded with fura-2 for 30 min with the following drugs present during loading at room temperature and the experimental period at 25 °C: (**a**) none; (**b**) 50 nM TG; (**c**) 20 μM TG; (**d**) 10 μM tBHQ; (**e**) 20 μM CPA. At the times shown by the arrow, KCl (final concn. 30 mM) was added. $[Ca^{2+}]_i$ was measured every 1200 ms for individual cells as described in the Materials and methods section.

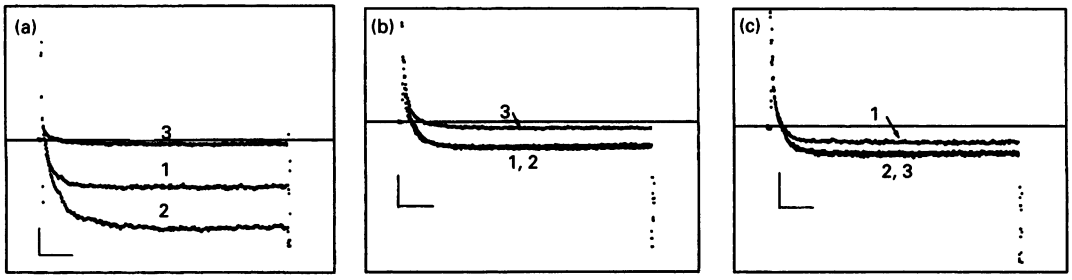

Figure 8 Effect of TG, tBHQ and CPA on whole-cell L-type Ca^{2+}-channel currents

Each drug (10 μM) was applied externally in the bath solution as described in the Materials and methods section. (**a**), (**b**) and (**c**) show current traces in response to a pulse to +10 mV from −40 mV holding potential before (trace 1), immediately after (trace 2) and 8 min after (trace 3) the application of TG, tBHQ and CPA respectively. Calibration bars in each panel represent 10 ms (horizontal) and 250 pA.

Table 1 Summary of the effects of tG, tBHQ and CPA on L-type Ca^{2+}-channel currents

The Table shows the normalized peak current values (mean ± S.E.M.) in the absence and presence of the three drugs over 8 min. Decreases in mean control current amplitude reflect channel run-down. *Significant difference from the respective control values at that time point ($P < 0.05$, $n = 4$–5). Numbers of experiments are given in parentheses.

Time (min)	Normalized current amplitude (arbitrary units)			
	Control	10 μM TG	10 μM tBHQ	10 μM CPA
0	1.0 ± 0 (5)	1.88 ± 0.18* (7)	1.08 ± 0.13 (5)	1.65 ± 0.34 (4)
5	0.81 ± 0.06 (5)	0.51 ± 0.19 (5)	0.15 ± 0.004* (5)	1.74 ± 0.33* (4)
8	0.68 ± 0.11 (5)	0.21 ± 0.02* (4)	0.06 ± 0.007* (4)	1.36 ± 0.18* (4)

The effect of drugs on Ca^{2+} currents was measured directly by using the whole-cell voltage-clamp configuration of the patch-clamp technique (Figure 8; Table 1). Addition of 10 μM CPA to the external bath solution caused a sustained increase in the Ca^{2+}-channel current amplitude over 8 min (Figure 8c; Table 1). On the other hand, 10 μM tBHQ caused a progressive inhibition of the Ca^{2+} current (Figure 8b; Table 1) and 10 μM TG caused a significant enhancement immediately after application, followed by a sustained block of the Ca^{2+} current, over 8 min (Figure 8a; Table 1). Ca^{2+}-channel current in the absence of any drug exhibits run-down over a period of minutes. Therefore, control experiments were performed wherein cells were perfused with drug-free solution and Ca^{2+}-channel current was measured over a period of 8 min, and the effect of drugs was compared with the control current amplitude at each time point (Table 1). None of the three drugs caused a shift in the relationship between peak Ca^{2+}-channel current and membrane potential.

DISCUSSION

TG, tBHQ and CPA all effectively blocked the initial Ca^{2+} spike elicited by TRH, consistent with their well-documented ability to deplete IP_3-releasable intracellular Ca^{2+} pools by blocking SERCA ATPases and previous observations in GH_3 cells [22,26]. TG was the most potent inhibitor, nearly abolishing the spike phase of the TRH response at 5 nM. tBHQ and CPA blocked the agonist-stimulated Ca^{2+} spike at 500 nM, concentrations that are well below those usually employed for this purpose, $\geqslant$ 10 μM. In isolated membranes, sub-micromolar concentrations of tBHQ and CPA and stoichiometric concentrations of TG are adequate to inhibit SERCA ATPases [6,9,10,14]. Low concentrations of tBHQ and CPA were probably effective in intact GH_3 cells because we employed a 30 min preincubation before challenging with TRH. TG forms a catalytically inactive dead-end complex with SERCA ATPases, binding to the form of the enzyme normally present in low Ca^{2+} and inhibiting formation of the enzyme–phosphate complex and other steps; tBHQ is believed to have a similar mechanism of action. When we added TG or tBHQ 1 min instead of 30 min before TRH, higher doses were required for complete inhibition of the agonist-induced Ca^{2+} spike (results not shown). This finding suggests that drug access to, or interaction with, the intracellular SERCA ATPases is rate-limiting, rather than the endogenous leak of Ca^{2+} from the endoplasmic reticulum, which would be unaffected by drug dose.

On the basis of cell-fractionation studies, Tanaka and Tashjian [33] have postulated three different intracellular Ca^{2+} pools: one sensitive to IP_3, a smaller compartment sensitive to TRH and also releasable with IP_3, and a larger caffeine-sensitive pool not sensitive to IP_3 but releasable with ionomycin. Recent evidence obtained with other cell types raises the possibility that multiple Ca^{2+} pools can be formed during cell fractionation from what appears to be a contiguous pool in the intact cell [34]. Regardless of whether intact GH_3 cells contain functionally distinct pools, it is clear that TRH cannot increase $[Ca^{2+}]_i$ after TG, tBHQ or CPA treatment.

When TRH was added to cells after depletion of the IP_3-releasable pool with any of the three inhibitors, the peptide often caused a pronounced decline in $[Ca^{2+}]_i$. Drummond [35] reported that $[Ca^{2+}]_i$ decreased when TRH was added to cells after high K^+. TRH stimulated the efflux of cytosolic Ca^{2+} even under conditions where $[Ca^{2+}]_i$ did not increase, as was the case after TG, tBHQ or CPA. Continuing studies indicate that this may be the result of direct stimulation of plasma-membrane Ca^{2+}-ATPases (E. J. Nelson and P. M. Hinkle, unpublished work).

Two of the three SERCA ATPase inhibitors, TG and tBHQ, showed an unexpected ability to inhibit the $[Ca^{2+}]_i$ response to depolarization and the influx of $^{45}Ca^{2+}$ through L-type voltage-operated Ca^{2+} channels. The inhibition of L-channel activity was not the indirect result of depleting intracellular Ca^{2+} pools, because it did not occur after treatment with even high concentrations of CPA. Since TG and tBHQ inhibited the Ca^{2+} current in patch-clamp studies, the drugs either acted directly at the channel or exerted their effects without the requirement for diffusible cytoplasmic components, which would have been dialysed away in the preparation.

The onset of Ca^{2+} channel inhibition by TG and tBHQ was slow, and 10–20 min was required to achieve maximal effects in intact cells. Inhibition by TG was not reversible over 2 h, but the effects of tBHQ on L-type channels were fully reversible, with depolarization-stimulated $^{45}Ca^{2+}$ uptake restored to control values 40 min after withdrawing the drug. Since TG is very hydrophobic, it may not have been effectively removed by simple wash-out. TG inhibition of SERCA ATPases is essentially irreversible, even though the complex is non-covalent [6,10,11,14]. The molecular mechanism of inhibition by tBHQ and TG of L-type Ca^{2+}-channel activity is not known, and is not likely to be similar to the mechanism of inhibition of the ATPases. Our experiments would not uncover effects on T-channels, which are also present in GH_3 cells, since these were inactivated at the holding potential of −40 mV in the patch-clamp studies and do not contribute much to overall $^{45}Ca^{2+}$ uptake.

Single-cell imaging permits spontaneous and agonist-induced $[Ca^{2+}]_i$ oscillations to be monitored. GH_3 cells display spontaneous $[Ca^{2+}]_i$ oscillations that are entirely dependent on the activity of L-type voltage-operated Ca^{2+} channels, since they can be blocked by L-channel blockers or removal of extracellular Ca^{2+} [22–25,29]. TG and CPA did not alter the characteristics of spontaneous Ca^{2+} oscillations at concentrations of 50 nM and 20 μM respectively, which are more than adequate to deplete IP_3-releasable pools. These results indicate that the spontaneous $[Ca^{2+}]_i$ transients in GH_3 pituitary cells do not require any contribution of Ca^{2+} from intracellular pools dependent on SERCA ATPases, consistent with published findings indicating that the oscillations are driven by spontaneous action potentials and result from Ca^{2+} influx through dihydropyridine-sensitive Ca^{2+} channels [22–27]. Consistent with this, TG eliminated spontaneous Ca^{2+} oscillations at 10 μM, where it blocked L-channel activity, whereas CPA did not interfere with the oscillations.

The dose–response curves for the two inhibitory effects of tBHQ, depletion of IP_3-sensitive stores and inhibition of L-channel activity, overlapped considerably. The lowest dose of tBHQ that blocked the initial $[Ca^{2+}]_i$ spike in response to TRH, 500 nM, also decreased the $[Ca^{2+}]_i$ response to depolarization by 50%. At least in the GH_3-cell model, tBHQ cannot be used to block agonist-stimulated $[Ca^{2+}]_i$ increases without inhibiting Ca^{2+} channels, and this previously unrecognized site of drug action may be a limitation to the use of tBHQ with any electrically excitable cell.

TG, on the other hand, had no effect on L-channel activity at concentrations sufficient to block completely agonist-evoked Ca^{2+} release, and with judicious choice of concentrations TG can be used without concern about effects on L-channel activity. TG has the disadvantage of its irreversibility, relatively high cost and propensity to stick to plasticware, making it difficult to wash out of cuvettes and microscope chambers.

CPA was an effective inhibitor of agonist-stimulated release of intracellular Ca^{2+}, and it did not inhibit L-channels. In fact, CPA caused a slight stimulation of $^{45}Ca^{2+}$ uptake through L-channels, and a small but significant increase in Ca^{2+}-channel current in patch clamp. The drug is inexpensive and relatively easy to wash away. Some cells respond to the depletion of intracellular stores with increased Ca^{2+} permeability at the plasma membrane, termed capacitative Ca^{2+} uptake [20]. CPA and tBHQ are both reported to interfere with the normal increase in Ca^{2+} permeability that follows depletion of intracellular stores in thymic lymphocytes [19]. Capacitative uptake appears to be a minor component in pituitary GH_3 cells at 25 °C, but may be more prominent at higher temperatures. Since CPA inhibits capacitative uptake only weakly and does so only at very high concentrations, 25–50 μM [19], it can probably be used safely at lower concentrations to deplete IP_3-releasable pools with little effect at channels involved in either voltage-dependent or capacitative uptake.

In summary, TG, tBHQ and CPA all inhibit SERCA ATPase and deplete agonist-releasable pools at sub-micromolar concentrations. TG and tBHQ also inhibit the activity of L-type voltage-operated Ca^{2+} channels. Depletion of IP_3-sensitive Ca^{2+} pools has no effect on spontaneous $[Ca^{2+}]_i$ oscillations in GH_3 cells. For TG, inhibition of Ca^{2+}-channel activity can be averted by selecting drug dose cautiously, but for tBHQ, inhibition occurs at the same concentrations as for inhibition of agonist mobilization of intracellular Ca^{2+}. This potential additional site of action of some SERCA ATPase inhibitors should be considered in interpreting results obtained with several useful and widely employed inhibitors.

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