Coupling between Intracellular Ca\(^{2+}\) Stores and the Ca\(^{2+}\) Permeability of the Plasma Membrane

COMPARISON OF THE EFFECTS OF THAPSIGARGIN, 2,5-DI-(TERT-BUTYL)-1,4-HYDROQUINONE, AND CYCLOPIAZONIC ACID IN RAT THYMIC LYMPHOCYTES*

(Received for publication, April 12, 1991)

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The regulation of Ca\(^{2+}\) uptake by receptors is incompletely understood. It has been proposed that the Ca\(^{2+}\) permeability of the plasma membrane increases in response to depletion of a critical intracellular Ca\(^{2+}\) storage compartment (Takemura, H., Hughes, A. R., Thastrup, O., and Putney, J. W. (1989) J. Biol. Chem. 264, 12266–12271). This hypothesis is based largely on the effect of thapsigargin, an inhibitor of endomembrane Ca\(^{2+}\)-ATPases. Due to the existence of an endogenous leak, inhibition of Ca\(^{2+}\) uptake by thapsigargin induces depletion of the stores. This is accompanied by increased plasmalemmal Ca\(^{2+}\) permeability, without change in the level of inositol phosphates. On the other hand, depletion of the intracellular stores by 2,5-di-(tert-butyl)-1,4-hydroquinone (BHQ), a chemically unrelated inhibitor of the Ca\(^{2+}\)-ATPases, fails to induce Ca\(^{2+}\) influx (Kass, G. E., Duddy, S. K., Moore, G. A., and Orrenius, S. (1989) J. Biol. Chem. 264, 15192–15198). In an attempt to reconcile these observations, we analyzed in lymphocytes the mode of action of thapsigargin and BHQ. In addition, we tested the effects of cyclopiazonic acid (CPA), a blocker of the skeletal muscle sarcoplasmic reticulum Ca\(^{2+}\)-ATPase. All three compounds released Ca\(^{2+}\) from a common intracellular compartment. Thapsigargin and low concentrations of BHQ and CPA concomitantly elevated the plasmalemmal Ca\(^{2+}\) permeability. Higher concentrations of BHQ and CPA produced a secondary inhibition of the Ca\(^{2+}\) entry pathway, by a mechanism seemingly unrelated to their effects on the internal stores. This inhibitory side effect can account for the reported discrepancies between the effects of thapsigargin and BHQ. The data provide further support for the notion that endomembrane Ca\(^{2+}\) stores are functionally coupled to the plasma membrane Ca\(^{2+}\) permeability pathway.

A variety of mechanisms have been postulated to account for the receptor-mediated activation of the plasmalemmal Ca\(^{2+}\) permeability by growth factors, hormones, or neurotransmitters. These include a direct coupling between the receptor and the transport pathway (Benham and Tsien, 1987), or coupling via second messengers such as inositol phosphates (Kuno and Gardner, 1987; Morris et al., 1987), cyclic AMP (Kelley et al., 1990), or cyclic GMP (Pandol and Schoefield-Payne, 1990). Alternatively, it has been proposed that the site of action of the second messengers is an intracellular Ca\(^{2+}\) storage compartment, which in turn determines the Ca\(^{2+}\) permeability of the plasma membrane. It is not clear how information is conveyed from the internal Ca\(^{2+}\) store to the surface membrane, but two mechanisms have been suggested. Some authors believe that the plasmalemmal Ca\(^{2+}\) permeability increases in response to the elevated [Ca\(^{2+}\)]\(_{i}\), which results from release of the endomembrane Ca\(^{2+}\) stores (Von Tscharner et al., 1986; Ng et al., 1988, 1990). Others have proposed that the plasma membrane is responsive to the Ca\(^{2+}\) content of the stores, regardless of the cytosolic Ca\(^{2+}\) concentration (Putney, 1986; Takemura et al., 1989). Support for the latter model has come from experiments designed to deplete intracellular Ca\(^{2+}\) pools by a method independent of receptor activation and inositol phosphate production. This can be accomplished with thapsigargin, a sesquiterpene lactone that is a potent and selective inhibitor of the microsomal Ca\(^{2+}\)-ATPase (Thastrup, 1990). Ostensibly due to a substantial endogenous leak, inhibition of the ATPase by the lactone produces a rapid release of Ca\(^{2+}\) from an endomembrane pool that includes the IP\(_{3}\)-sensitive compartment (Thastrup et al., 1990). In a variety of cells, the addition of thapsigargin also results in the concomitant elevation of plasma membrane Ca\(^{2+}\) permeability (Thastrup, 1990). These findings are consistent with the "capacitative" coupling model, which stipulates that the plasmalemmal permeability is controlled by the degree of filling of an intracellular Ca\(^{2+}\) storage compartment. Although attractive, the capacitative model has thus far failed to explain certain observations. In murine cells of the neural line NG115-401L, exposure to thapsigargin induces depletion of the endomembrane stores, yet is not accompanied by an increased Ca\(^{2+}\) permeability of the plasma membrane (Jackson et al., 1988). Moreover, discordant results have been reported using a different inhibitor of the microsomal Ca\(^{2+}\) ATPase. BHQ, a synthetic compound chemically unrelated

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1 The abbreviations used are: [Ca\(^{2+}\)]\(_{i}\), cytosolic free calcium concentration; IP\(_{3}\), inositol 1,4,5-trisphosphate; BHQ, 2,5-di-(tert-butyl)-1,4-hydroquinone; AM, acetoxymethyl; dimethyl-BAPTA, 5,5'-di-methyl-1,2-bis-(2-aminoephenoxy)ethane-N, N',N'-tetraacetic acid; EGTA, ethylenbis(oxymethyleneenitrilo)tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; THG, thapsigargin; CPA, cyclopiazonic acid; DTPA, diethyltestraminepentacetic acid.

2 2,5-Di-(tert-butyl)-1,4-hydroquinone (BHQ) is also referred to in the literature as 2,5-di-(tert-butyl)-1,4-benzohydroquinone.

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* This work was supported by the Medical Research Council of Canada and the National Cancer Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a postdoctoral fellowship of the Medical Research Council of Canada.

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Vol. 266, No. 31, Issue of November 5, pp. 20856-20862, 1991
Printed in U.S.A.
to thapsigargin, effectively depletes internal Ca\(^{2+}\) stores in hepatocytes, without increasing the plasmalemmal Ca\(^{2+}\) permeability (Kass et al., 1989). Unlike the results in NG115–401L cells, the latter observation cannot be attributed to tissue specificity, since thapsigargin effectively increased Ca\(^{2+}\) influx in hepatocytes (Thastrup et al., 1990; Thastrup, 1990). Thus, the source of this discrepancy remains unclear, questioning the validity of the capacitative model. In an attempt to reconcile the results obtained with thapsigargin and BHQ, we have undertaken a systematic study of the mode of action of these two inhibitors, under otherwise identical conditions. In addition, we also tested the effects of cyclopiazonic acid (CPA), an inhibitor of the Ca\(^{2+}\)-ATPase of skeletal muscle sarcoplasmatic reticulum (Siedler et al., 1989). Because of the similarities between the Ca\(^{2+}\)-ATPases of the endoplasmic and sarcoplasmatic reticuli, we anticipated that CPA would effectively deplete intracellular Ca\(^{2+}\) stores in non-muscle cells. The experiments were carried out in rodent lymphocytes, which have been reported to respond to thapsigargin with increased plasma membrane Ca\(^{2+}\) permeability (Mason et al., 1991). Our results indicate that all three compounds effectively deplete the stores and promote Ca\(^{2+}\) influx. At concentrations higher than those required for depletion of the stores, BHQ and, to a lesser extent, CPA inhibit the entry of Ca\(^{2+}\) across the plasma membrane. Therefore, the apparent inconsistency of earlier reports can be accounted for by a secondary effect of some of the inhibitors, lending further credence to the capacitative model.

**MATERIALS AND METHODS**

**Reagents and Solutions**

The AM derivatives of indo-1 and dimethyl-BAPTA were purchased from Molecular Probes (Eugene, OR). Ionomycin and bovine serum albumin (Fraction V; fatty acid, nucleos- and protease-free) were obtained from Calbiochem Behring. The culture medium RPMI 1640 (bicarbonate-free), EGTA, HEPES, and CPA were purchased from Sigma. Thapsigargin was purchased from LC Services Corp. (Woburn, MA). BHQ, DTPA, and anhydrous dimethyl sulfoxide were obtained from Aldrich. NaCl, KCl, CaCl\(_2\), MgCl\(_2\), MnCl\(_2\), Mg- and K-glucose, NaOH, KOH, and dibutyl phthalate were purchased from Fisher Scientific. LaCl\(_3\) was from BDH Chemicals Ltd. (Poole, U. K.). CaCl\(_2\) was obtained from ICN Radiochemicals (Irvine, CA), and the scintillation mixture Aquasol-2 from Du Pont-New England Nuclear. Stock solutions of Indo-1 AM, dimethyl-BAPTA-AM, thapsigargin, CPA, and BHQ were made up in anhydrous dimethyl sulfoxide. Ionomycin was dissolved in ethanol.

The basic Na\(^+\) solution contained 140 mM NaCl, 3 mM KCl, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), 0.2 mM EGTA, 10 mM d-glucose, and 20 mM HEPES-free acid, plus 1 mg/ml albumin. The solution was titrated to pH 7.25 at 37 °C with NaOH. Ca\(^{2+}\)-free solution was made by omitting Ca\(^{2+}\) and increasing the EGTA concentration to 0.5 mM. When required, Mn\(^{2+}\) was added directly to the solution as MnCl\(_2\).

The osmolarity of the solution was adjusted to 295–300 mosm prior to the addition of albumin using an Osmette freezing point osmometer. All solutions and stocks were stored at −20 °C.

**Cell Isolation and Manipulations**

Thymic lymphocytes were isolated from 140–200-g male Wistar rats (The Charles River Breeding Laboratories) as previously described (Grinstein et al., 1984). The cells were counted using a Model C M Coulter Counter (Coulter Electronics, Hialeah, FL) and maintained at room temperature (37°C) in bicarbonate-free RPMI 1640 culture medium buffered to pH 7.4 with 20 mM HEPES.

**Fluorescence Determinations**

All experiments were performed at 37 °C using a Hitachi Model F-4000 fluorescence spectrophotometer equipped with a magnetic stirrer. The cells were counted immediately after the last manipulation, prior to addition to the cuvette, to ensure that the appropriate cell number was added.

**Calcium Permeability of Lymphocytes**

**Determination of Free Cytosolic Calcium Concentration—[Ca\(^{2+}\)\(^{40+}\).** was determined by measuring the fluorescence of Indo-1. The excitation and emission wavelengths used were 331 nm (3-nm slit width) and 410 nm (10-nm slit), respectively. Thymocyte suspensions (25 × 10\(^6\) cells/ml) were loaded with indo-1 by incubation with a 2 μM concentration of the AM form of the indicator Indo-C1000 for 30 min at 37°C in basic solution devoid of albumin and EGTA. The cells were then sedimented, resuspended in basic Na\(^+\) solution devoid of albumin and EGTA, and kept at room temperature until required. To monitor fluorescence, aliquots containing the required cell number were sedimented, resuspended in Na\(^+\) solution plus or minus Ca\(^{2+}\), as indicated, and added to the cuvette. The fluorescence of indo-1 was calibrated using ionomycin and Mn\(^{2+}\) as previously described (MacDougal et al., 1988). A dissociation constant of 250 nM for the indo-1-Ca\(^{2+}\) complex was used to calculate [Ca\(^{2+}\)]. (Gryniewicz et al., 1985).

**Determination of Mn\(^{2+}\) Influx—Mn\(^{2+}\) uptake was monitored as the rate of quenching of indo-1 fluorescence measured at the isoeosopic point for the Ca\(^{2+}\)-indo-1 complex (excitation 345 nm, emission 455 nm). When measured at the isoeosopic wavelengths, the rate of fluorescence decrease is insensitive to changes in [Ca\(^{2+}\)] and is proportional to the rate of Mn\(^{2+}\) accumulation in the cytosol. Similar techniques have been employed for the measurement of Mn\(^{2+}\) uptake by platelets, neutrophils, and endothelial cells using fura2 and quin2 (Hallam and Rink, 1986; Merritt et al., 1989; Hallam et al., 1989).

**“Ca\(^{2+}\)” Influx Determinations**

To mimic the conditions used for the determination of [Ca\(^{2+}\)], 3 × 10\(^6\) cells were loaded with 2 μM indo-1-AM at a concentration of 25 × 10\(^6\) cells/ml for 25 min at 37°C in basic experimental solution devoid of albumin and EGTA. To prolong the linear phase of unidirectional “Ca\(^{2+}\)” uptake, cells were simultaneously loaded with dimethyl-BAPTA by adding a 4 μM concentration of the AM precursor to this incubation medium. 2.5 × 10\(^6\) cells were then sedimented and resuspended in 0.65 ml of basic experimental solution. A 0.6-ml aliquot of the suspension was transferred to a 1.5-ml microcentrifuge tube and maintained at 37°C, while the remaining 0.05 ml was used for cell number determinations. Measurement of Ca\(^{2+}\) uptake was initiated by addition of “Ca\(^{2+}\)” (10 μCi/ml final concentration). Uptake was terminated after the required interval by transferring 0.1-ml aliquots of the cell suspension to 1.0 ml of ice-cold Na\(^{+}\)-free solution devoid of EGTA and containing 0.1 ml of LaCl\(_3\), followed by centrifugation through an oil phase composed of 2 parts vegetable oil (Mazola) and 10 parts dibutyl phthalate (v/v). Triplicate samples of the supernatant were counted to ascertain the specific activity. The supernatant was then removed from above the oil layer, the tip of the tube containing the pellet was cut and placed in a scintillation vial, and the pellet was lysed by the addition of 0.5 ml of distilled water. Following the addition of 10 ml of Aqausol-2, the samples were counted by liquid scintillation (LKB Rackbeta, Turku, Finland).

Unless otherwise indicated, all experiments were performed at 37°C using the results and number of preparations indicated in parentheses. The fluorescence traces illustrated are representative of experiments in a minimum of three preparations.

**RESULTS**

The ability of thapsigargin to alter cytosolic [Ca\(^{2+}\)], in rat thymic lymphocytes was examined using the fluorescent Ca\(^{2+}\) indicator indo-1. In the presence of external Ca\(^{2+}\), thapsigargin induced a sustained dose-dependent rise in [Ca\(^{2+}\)], as illustrated in Fig. 1, A and B. Similar increases in [Ca\(^{2+}\)] during thapsigargin exposure were previously reported in human peripheral T-lymphocytes (Scharff et al., 1988) and in the human T-cell clone P28 (Gouy et al., 1990). In the absence of external Ca\(^{2+}\), exposure of thymocytes to thapsigargin produced a transient increase in [Ca\(^{2+}\)] (Fig. 1C). In other cell types, similar transient increases at a time points are known to be unmasking of an endogenous Ca\(^{2+}\) leak from the endomembrane pool upon inhibition of the Ca\(^{2+}\)-ATPase (Thastrup, 1990; Thastrup et al., 1990). The dose dependence of the rise in [Ca\(^{2+}\)] induced by thapsigargin in the presence or absence of extracellular Ca\(^{2+}\) is summarized in Fig. 1D. In the presence of external Ca\(^{2+}\), maximally effective concentrations of thapsigargin elevated [Ca\(^{2+}\)], by 1079 ± 217 nM (n = 4), from a
resting level of 190 ± 9 nM (n = 24). It is noteworthy that, under these conditions, the rise in [Ca2+]i was sustained at all concentrations of thapsigargin tested. The efficacy of the thapsigargin-induced release of Ca2+ from endomembrane pools, as measured by the transient rise in [Ca2+]i, in the absence of external Ca2+, is paralleled by the ability of the lactone to generate the sustained [Ca2+]i increase in the presence of external Ca2+. Maximal responses were observed in both cases at thapsigargin concentrations between 3 and 30 nM. In the absence of external Ca2+, a maximal dose of thapsigargin resulted in a [Ca2+]i transient peaking at 89 ± 11 nM (n = 4) above the resting level.

An identical analysis of the effect of BHQ on [Ca2+]i was performed. The release of Ca2+ from intracellular stores induced by BHQ showed a pattern similar to that observed with thapsigargin (cf. Figs. 2C and 1C). In the range studied, the Ca2+ released by BHQ increased monotonically with the concentration of the inhibitor, reaching a maximum of 94 ± 9 nM (n = 3) above the basal level between 5 and 25 μM BHQ (Fig. 2D). In contrast, the effect of BHQ in the presence of extracellular Ca2+ differed markedly from that of thapsigargin. While low doses of BHQ induced a sustained [Ca2+]i increase (Fig. 2B), the change in [Ca2+]i in the presence of high doses of BHQ (e.g. 50 μM) was biphasic. A rapid rise in [Ca2+]i was followed by a secondary decline toward the resting level (Fig. 2A). When the former (peak) phase is plotted as a function of the BHQ concentration, two points become apparent (Fig. 2D). First, the [Ca2+]i rise induced by 50 μM BHQ is smaller than that attained with 10 or 25 μM. This effect cannot be attributed to an inability of higher concentrations of BHQ to inhibit the endomembrane ATPase, as indicated by the results obtained in the absence of extracellular Ca2+ (see above). Second, the maximal change in [Ca2+]i, in the presence of external Ca2+, is much lower than that induced by thapsigargin (389 ± 27 nM (n = 4) for BHQ versus 1079 nM for thapsigargin). Taken together, these data suggest that the biphasic increase in [Ca2+]i, observed in the presence of external Ca2+ (Fig. 2A) and the biphasic nature of the dose-response curve generated under similar conditions (open symbols in Fig. 2D) are the result of a secondary effect of high concentrations of BHQ, unrelated to its ability to inhibit the endomembrane ATPase.

Using experiments like those outlined above, we also investigated the effect of a third endomembrane ATPase inhibitor, CPA, on [Ca2+]i, homeostasis. Cells suspended in Ca2+-free solution and exposed to CPA demonstrated a transient increase in [Ca2+]i. This response, which resembles the effects obtained with thapsigargin and BHQ under comparable conditions, is indicative of release of Ca2+ from an intracellular store (Fig. 3C) and suggests that CPA impaired the endomembrane Ca2+-ATPase. Ca2+ release from stores was maximal at ≈5 μM CPA, and comparable responses were obtained with higher concentrations (Fig. 3D). In the presence of external Ca2+, low doses of CPA induced a sustained increase in [Ca2+]i, (Fig. 3B). Higher concentrations of CPA (50 μM) elicited a biphasic change in [Ca2+]i: a large initial increase
followed by a slow recovery toward basal levels. This secondary recovery phase was slower than that seen in the presence of high concentrations of BHQ (cf. Figs. 2A and 3A) and, unlike the latter, was not consistently observed.

A detailed analysis of the concentration dependence of the effects of CPA is summarized in Fig. 3D. Like thapsigargin, the efficacy of the CPA-induced release of Ca²⁺ from endomembrane pools, as measured by the transient rise in [Ca²⁺], in the absence of external Ca²⁺, is closely paralleled by its ability to induce the larger, more sustained change in the presence of external Ca²⁺. Maximal responses in both the presence and absence of external Ca²⁺ were observed at a concentration between 5 and 10 µM CPA. In the presence of external Ca²⁺, maximally effective concentrations of CPA resulted in a peak increase in [Ca²⁺] of 549 ± 49 nM (n = 6), which is intermediate between the responses obtained with thapsigargin (1079 nM) and BHQ (289 nM). In the absence of external Ca²⁺, a maximal dose of CPA results in a [Ca²⁺], transient which peaks at a value 93 ± 11 nM (n = 3) above the resting level. This peak increase in [Ca²⁺], is virtually identical with that induced by BHQ and thapsigargin (94 nM and 59 nM, respectively).

Although it is apparent that thapsigargin, BHQ, and CPA release comparable amounts of intracellular Ca²⁺, it is not clear whether the same pool is affected in every instance. It is therefore conceivable that the variable rise in [Ca²⁺], induced by the different inhibitors in Ca²⁺-containing medium reflects the involvement of different stores, which exert variable degrees of control over the plasmalemmal Ca²⁺ permeability. To investigate the degree of overlap between the stores released by thapsigargin, BHQ, and CPA, we monitored the changes in indo-1 fluorescence during sequential addition of the inhibitors to cells suspended in Ca²⁺-free solution. While the addition of 50 µM BHQ resulted in a transient rise in [Ca²⁺], a subsequent addition of 30 nM thapsigargin was found to have no effect (Fig. 4A). If the order of presentation was reversed, thapsigargin induced a marked transient increase in [Ca²⁺], with the secondary addition of BHQ having no detectable effect (Fig. 4B). These findings suggest that the Ca²⁺ pools depleted by both inhibitors overlap extensively. A similar pattern emerged when combinations of thapsigargin and CPA were investigated (Fig. 4, C and D). While 50 µM CPA induced a transient rise in [Ca²⁺], a subsequent addition of 30 nM thapsigargin was without effect (Fig. 4C). Conversely, CPA addition was without effect if added after thapsigargin (Fig. 4D). Control experiments ruled out the possibility that the lack of effect of the second inhibitor was due to the depletion of the intracellular Ca²⁺ pool, as a result of the prolonged (approximately 10-min) incubation in Ca²⁺-free solution required to assess the effects of the first inhibitor. While some depletion does occur during this interval, a clearcut transient increase in Ca²⁺ can still be induced by thapsigargin addition following a 14-min incubation in Ca²⁺-free solution, provided no ATPase inhibitor is added previously (results not shown). Taken together, these results are consistent with the notion that thapsigargin, BHQ, and CPA release Ca²⁺ from an identical intracellular pool.

In view of these findings, the differential effect of the inhibitors on [Ca²⁺], in Ca²⁺-containing medium cannot be attributed to variable degrees of depletion of a critical Ca²⁺ store. We therefore considered the possibility that BHQ and, to a lesser extent, CPA reduce the level of [Ca²⁺], by either interfering with Ca²⁺ uptake at the plasma membrane or by accelerating its extrusion. To test this possibility, cells were initially treated with 50 µM BHQ and subsequently exposed to thapsigargin (Fig. 5A). As described above, this concentration of BHQ produced a biphasic change in [Ca²⁺], with a secondary sustained increase to ~300 nM. When added after BHQ, thapsigargin was without effect. In parallel experiments performed with the same batch of cells (Fig. 5B), thapsigargin alone increased [Ca²⁺], to values in excess of 800 nM. This implies that BHQ inhibited the effect of thapsigargin on [Ca²⁺], This can be demonstrated more clearly by reversing the order of addition of the reagents (Fig. 5C). When added after [Ca²⁺], was maximally elevated by thapsigargin, BHQ produced a precipitous drop in [Ca²⁺], to levels similar to those found in cells treated with BHQ alone.

These observations are consistent with partial inhibition

FIG. 3. Effect of CPA on [Ca²⁺]. The cells were suspended in Na⁺ solution containing 1 mM Ca²⁺ and 0.2 mM EGTA (panels A and B) or in a Ca²⁺-free solution containing 0.5 mM EGTA (panel C), as in Fig. 1. CPA, at the concentrations indicated, was added at the arrowhead. D, summary of the effects of increasing CPA concentrations on the maximal rise in [Ca²⁺], measured in the presence (+ Ca²⁺) or absence (−Ca²⁺) of extracellular Ca²⁺. Data points were collected from experiments like those in A-C and are the mean ± S.D. of results from a minimum of three preparations.

FIG. 4. Additivity of the effects of thapsigargin, BHQ, and CPA on the release of Ca²⁺ from intracellular stores. Cells were loaded with indo-1 in Ca²⁺-containing medium and suspended in Ca²⁺-free medium, and the fluorescence was monitored. Thapsigargin (30 nM), BHQ (50 µM), or CPA (50 µM) was added where indicated. The traces are representative of a minimum of three similar experiments.
by BHQ of the activated plasma membrane Ca\(^{2+}\) influx pathway. Alternatively, BHQ may stimulate Ca\(^{2+}\) extrusion from the cell or activate sequestration of Ca\(^{2+}\) into an inhibitor-resistant intracellular pool. To assess the first possibility, we determined the effect of the inhibitors on the rate of unidirectional Ca\(^{2+}\) influx, measured isotopically. To minimize backflux, thereby prolonging the linear phase of Ca\(^{2+}\) uptake, the cytosolic Ca\(^{2+}\) buffering power was increased by loading the cells with dimethyl-β-APTA. The results are summarized in Fig. 6. Addition of 300 nM thapsigargin stimulated the rate of Ca\(^{2+}\) uptake approximately 10-fold, from the resting value of 20 ± 3 pmol/min to 207 ± 34 pmol/min (n = 3). Uptake was also stimulated by BHQ and by CPA, but to a considerably lower extent. The rates attained averaged 87 ± 13 pmol/min and 88 ± 3 pmol/min (n = 3), respectively. The reduced potency of CPA and BHQ to stimulate Ca\(^{2+}\) influx parallels their smaller effect on [Ca\(^{2+}\)]\(_{i}\), and suggests that these agents interfere with the Ca\(^{2+}\) entry pathway.

This conclusion was verified by measuring the unidirectional uptake of Mn\(^{2+}\), a Ca\(^{2+}\) surrogate, into indo-1-loaded thymocytes. Mn\(^{2+}\), which has been used successfully as a probe of Ca\(^{2+}\) influx pathways in platelets, neutrophils, and endothelial cells (Hallam and Rink, 1985; Merritt et al., 1989; Hallam et al., 1989), enters unstimulated thymocytes, resulting in gradual quenching of the fluorescent dye. The rate of fluorescence decrease, measured at the Ca\(^{2+}\) isosbestic point, provides a relative measure of the divalent cation permeability. Subsequent chelation of extracellular Mn\(^{2+}\) with DTPA halts this decline, but no fluorescence recovery can be observed (not shown), indicating that Mn\(^{2+}\) is not extruded from the cells. This reflects the inability of the Ca\(^{2+}\) pump to transport Mn\(^{2+}\), rather than slow dissociation of the Mn\(^{2+}\)-indo-1 complex, since rapid fluorescence recovery was induced under these conditions by the addition of ionomycin. These findings validate the use of indo-1 quenching by Mn\(^{2+}\) as a discriminating measure of unidirectional divalent cation uptake in lymphocytes.

As shown in Fig. 7A, the rate of indo-1 quenching produced by Mn\(^{2+}\) was greatly accelerated by the addition of 30 nM thapsigargin, revealing the activation of a divalent cation permeability pathway in the plasma membrane. Subsequent addition of ionomycin, which can transport Mn\(^{2+}\) effectively, rapidly abolished the remaining fluorescence. At low concentrations (≤10 μM), BHQ also produced a sustained acceleration of the entry of Mn\(^{2+}\) (Fig. 8). In contrast, a biphasic effect was recorded at 50 μM BHQ: a rapid increase in the rate of quenching was followed by a return toward the basal rate (Fig. 8). The latter most likely reflects the inhibitory effect of high doses of BHQ on the divalent cation entry pathway. In support of this notion, we found that addition of 50 μM BHQ to cells previously stimulated with thapsigargin led to an abrupt decline in the rate of indo-1 quenching (91 ± 5%, n = 10; Fig. 7B). The inhibitory effect of BHQ could be readily bypassed by addition of ionomycin (Figs. 7B and 8).
The purpose of the present experiments was to resolve the apparent discrepancy between the reported effects of two putative inhibitors of the endomembrane Ca\(^{2+}\)-ATPase, thapsigargin and BHQ, on plasmalemmal Ca\(^{2+}\) permeability. In addition, we tested if CPA, a blocker of the ATPase of muscle sarcoplasmic reticulum, also precluded Ca\(^{2+}\) accumulation by the reticulum of non-muscle, lymphoid cells and whether plasmalemmal Ca\(^{2+}\) permeability was consequently affected. We found that, at the appropriate concentrations, all three compounds triggered the rapid release of intracellular Ca\(^{2+}\) stores. The same endomembrane storage pool was seemingly affected by the three agents, since their effects were not additive (Fig. 4). Although we made no attempt to confirm or investigate the exact mechanism responsible for the release of Ca\(^{2+}\) from intracellular stores induced by these inhibitors, preliminary evidence suggests that elevation of IP3 levels is not involved. We have been unable to detect increases in IP3 levels in rat thymic lymphocytes exposed to 300 nM thapsigargin, 50 \(\mu\)M BHQ, or 50 \(\mu\)M CPA. In the same experiments, the mitogenic lectin concanavalin A (20 \(\mu\)g/ml), which induces the release of Ca\(^{2+}\) from intracellular stores and a concomitant influx of extracellular Ca\(^{2+}\), increased IP3 levels approximately 6-fold (results not shown). These findings are in accordance with published reports that thapsigargin does not induce phophoinositide hydrolysis in the cultured T-cell line P28 (Gouy et al., 1990), parotid acinar cells (Takemura et al., 1989), or the neuronal cell line NG115–401 (Jackson et al., 1988). Similarly, BHQ failed to increase phosphoinositide hydrolysis in isolated rat hepatocytes (Kass et al., 1989). Thus, inhibition of the Ca\(^{2+}\)-ATPase and subsequent efflux of Ca\(^{2+}\) through a constitutive "leak" pathway is the likely mechanism underlying the observed depletion of the stores.

In addition to releasing Ca\(^{2+}\) from a common intracellular store, thapsigargin, BHQ, and CPA also promoted an increase in the divalent cation permeability of the plasma membrane as measured by: 1) a relatively sustained rise in [Ca\(^{2+}\)], in the presence, but not in the absence, of extracellular Ca\(^{2+}\); 2) an increase in the rate of 45Ca\(^{2+}\) uptake; and 3) an increase in the rate of Mn\(^{2+}\) influx, determined fluorimetrically. These findings are in good agreement with the model proposed by Putney and co-workers (Putney, 1986; Takemura et al., 1989), whereby the Ca\(^{2+}\) permeability of the plasma membrane is dictated by the degree of filling of an endomembrane Ca\(^{2+}\) storage compartment. While this model received strong support from the observations made with thapsigargin, it was difficult to rule out that this lactone had independent, direct effects on plasmalemmal permeability. The ability of BHQ and CPA to enhance Ca\(^{2+}\) entry into the cells provides further support for Putney's coupling model. These compounds, which are structurally unrelated to thapsigargin, share with this lactone the ability to deplete endomembrane Ca\(^{2+}\) stores. The relationship between these events is suggested by the parallel concentration dependence of the depletion of the stores (estimated in Ca\(^{2+}\)-free medium) and the entry of divalent cations (revealed by the difference in [Ca\(^{2+}\)], attained in the presence and absence of external Ca\(^{2+}\)), particularly at low doses of the drugs. The divergence noted at higher concentrations, which is due to a secondary effect on the plasma membrane, is discussed in more detail below. Increased plasmalemmal permeability to divalent cations in response to CPA and BHQ was confirmed using Mn\(^{2+}\).

While the above observations are compatible with the revised capacitive model, we cannot formally rule out the possibility that thapsigargin, BHQ, and CPA all directly activate Ca\(^{2+}\) uptake at the plasma membrane independently of their effect on the internal stores. This would require the presence of a plasmalemmal receptor for the drugs with an affinity similar to that of the endomembrane Ca\(^{2+}\)-ATPase. The increase in [Ca\(^{2+}\)], promoted by this putative receptor would involve a mechanism other than simple inhibition of the plasma membrane Ca\(^{2+}\)-ATPase, since the unidirectional influx of 45Ca\(^{2+}\) and the uptake of Mn\(^{2+}\) (which is not pumped out of the cells) are increased by the endomembrane pump inhibitors.

While at low concentrations all three inhibitors induced a sustained rise in [Ca\(^{2+}\)], a secondary decline in [Ca\(^{2+}\)], was observed in cells treated with higher concentrations of BHQ and CPA. In addition, the rise in [Ca\(^{2+}\)], induced by CPA and BHQ was markedly lower than that observed in the presence of thapsigargin. Since maximally effective concentrations of these compounds completely depleted the intracellular store(s), the differences in plasma membrane divalent cation permeability cannot be attributed to variable degrees of store depletion. Instead, inhibition of the plasma membrane influx pathway, or uncoupling of stores and the plasma membrane, could account for the smaller increase in [Ca\(^{2+}\)], induced by BHQ and CPA. The following evidence supports the conten-
tion that BHQ and to a lesser extent CPA have a direct inhibitory effect on the plasma membrane divalent cation permeability. First, pretreatment of cells with BHQ precluded the stimulation of Ca\(^{2+}\) entry by thapsigargin (Fig. 5A). Second, addition of BHQ after stimulation with thapsigargin reduced [Ca\(^{2+}\)]\text{c}\). (Fig. 5C). Third, BHQ and CPA inhibited the influx of Mn\(^{2+}\) activated by thapsigargin (Figs. 8 and 9). Therefore, it appears that low doses of BHQ and CPA deplete internal stores and increase plasmalemmal permeability, whereas higher concentrations tend to inhibit Ca\(^{2+}\) entry from the medium. This dual effect readily explains the biphasic nature of the [Ca\(^{2+}\)]\text{c}\), changes recorded at high concentrations of these agents and the lower [Ca\(^{2+}\)]\text{c}\), levels attained at maximally stimulatory doses of BHQ and CPA, compared with optimal doses of thapsigargin.

The ability of high concentrations of BHQ to release Ca\(^{2+}\) from intracellular stores while simultaneously inhibiting the uptake of Ca\(^{2+}\) across the plasma membrane can also explain the apparent discrepancy that exists regarding the regulation of the plasma membrane Ca\(^{2+}\) permeability in hepatocytes. In these cells, BHQ was reported to effectively deplete the internal stores without inhibitory effect on the uptake pathway, accounting for their failure to detect increased divalent cation entry.

It is apparent from the results presented that thapsigargin is the drug of choice for the unambiguous assessment of the effects of depletion of internal Ca\(^{2+}\) stores. In our experiments, concentrations of thapsigargin 10-fold larger than those required to deplete the stores were without inhibitory effect on Ca\(^{2+}\) or Mn\(^{2+}\) entry from the medium. However, caution must nevertheless be exercised, since preliminary experiments in leukocytes indicate that inhibition becomes significant when micromolar concentrations of thapsigargin are used. Because such high concentrations are required for effective depletion of stores in some tissues (Gouy et al., 1990; Thastrup et al., 1987; Jackson et al., 1988; Takemura et al., 1989), secondary effects cannot be ruled out and should be considered. It is also noteworthy that CPA and particularly BHQ are considerably less expensive than thapsigargin and may be suitable for certain studies, particularly when used at lower concentrations.

In summary, the results presented here provide a means to reconcile the data obtained in other cells with thapsigargin and BHQ. In addition, CPA is introduced as an alternative agent capable of depleting intracellular Ca\(^{2+}\) stores in intact non-muscle cells. Together, the actions of these three compounds support the contention that the degree of filling of the stores plays a determinant role in controlling Ca\(^{2+}\) entry into the cell, in accordance with Putney's revised capacitative model (Takemura et al., 1989).

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