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Depletion of the inositol 1,4,5-trisphosphate-sensitive intracellular Ca^{2+} store in vascular endothelial cells activates the agonist-sensitive Ca^{2+}-influx pathway

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Previous studies in non-excitable cells have suggested that depletion of internal Ca^{2+} stores activates Ca^{2+} influx from the extracellular space via a mechanism that does not require stimulation of phosphoinositide hydrolysis. To test this hypothesis in vascular endothelial cells, the effect of the Ca^{2+}-ATPase/pump inhibitor 2,5-di-t-butylhydroquinone (BHQ) on cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was examined. BHQ produced a dose-dependent increase in $[Ca^{2+}]_i$, which remained elevated over basal values for several minutes and was substantially inhibited in the absence of extracellular Ca^{2+}. Application of bradykinin after BHQ demonstrated that the BHQ-sensitive compartment partially overlapped the bradykinin-sensitive store. Similar results were obtained with thapsigargin and cyclopiazonic acid, two other Ca^{2+}-ATPase inhibitors. Although BHQ had no effect on phosphoinositide hydrolysis, both $^{45}Ca^{2+}$ influx and efflux were stimulated by this agent. These results suggest that depletion of the agonist-sensitive Ca^{2+} store is sufficient for activation of Ca^{2+} influx. Several characteristics of the Ca^{2+}-influx pathway activated by internal store depletion were compared with those of the agonist-activated pathway. Bradykinin-stimulated Ca^{2+} influx was increased at alkaline extracellular pH (pH_o), and was inhibited by extracellular La^{3+}, by depolarization of the membrane, and by the novel Ca^{2+}-influx blocker 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride (SKF 96365). Additionally, bradykinin stimulated influx of both $^{45}Ca^{2+}$ and $^{133}Ba^{2+}$, consistent with the hypothesis that the agonist-activated influx pathway is permeable to both of these bivalent cations. Likewise, activation of Ca^{2+} influx by BHQ, thapsigargin and cyclopiazonic acid was blocked by La^{3+}, membrane depolarization and SKF 96365, but was unaffected by nitrendipine or BAY K 8644. Furthermore, Ca^{2+} influx stimulated by BHQ was increased at alkaline pH_o and BHQ stimulated the influx of both $^{45}Ca^{2+}$ and $^{133}Ba^{2+}$ to the same extent. These results demonstrate that the agonist-activated Ca^{2+}-influx pathway and the pathway activated by depletion of the agonist-sensitive internal Ca^{2+} store are indistinguishable.

INTRODUCTION

An increase in the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of the vascular endothelial cell produced by vasoactive agents is necessary and sufficient for the production and release of paracrine factors which influence the tone of subjacent smooth-muscle cells. Previous studies have revealed that agonist agents such as bradykinin produce a well-characterized biphasic increase in $[Ca^{2+}]_i$ (Lückhoff & Busse, 1986; Hallam & Pearson, 1986; Colden-Stanfield *et al.*, 1987; Morgan-Boyd *et al.*, 1987; Schilling *et al.*, 1988; Hallam *et al.*, 1988). The initial transient component reflects Ins(1,4,5)P_3-induced release of Ca^{2+} from internal stores, whereas the sustained component of the $[Ca^{2+}]_i$ response reflects an agonist-induced activation of Ca^{2+} influx from the extracellular space (Colden-Stanfield *et al.*, 1987; Schilling *et al.*, 1988). Although the mechanisms involved in the release of Ca^{2+} from internal stores have been widely studied in a variety of non-excitable cells, electrophysiological identification and characterization of the influx pathway have been difficult. Several characteristic features of the pathway are, however, known from indirect studies. First, the influx pathway can be blocked by several inorganic bi- and ter-valent cations, including Ni^{2+} and La^{3+}, which produce inhibition in the mM and μM ranges respectively (Colden-Stanfield *et al.*, 1987; Pandol *et al.*, 1987; Schilling *et al.*, 1988; Kwan *et al.*, 1990; Buchan & Martin, 1991). Second, membrane depolarization by elevation of extracellular K^+ or by application of current via a patch-clamp pipette results in attenuation of agonist-activated Ca^{2+} influx in vascular endothelial cells (Sauve *et al.*, 1988; Schilling *et al.*, 1989; Schilling, 1989; Lückhoff & Busse, 1990*a*,*c*) and in several other non-excitable cell types (Altin *et al.*, 1988; Merritt & Rink, 1987; DiVirgilio *et al.*, 1987; Mohr & Fewtrell, 1987; Sage & Rink, 1986; Oettgen *et al.*, 1985). Third, the agonist-induced influx of Ca^{2+} is insensitive to the organic inhibitors or activators (e.g. nitrendipine or BAY K 8644) of voltage-gated Ca^{2+} channels found in nerve and muscle (Colden-Stanfield *et al.*, 1987; Schilling *et al.*, 1988). Fourth, a novel synthetic compound, 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride (SKF 96365) has been shown to block the agonist-induced changes in $[Ca^{2+}]_i$ in endothelial cells, platelets, and neutrophils (Merritt *et al.*, 1990). Fifth, recent studies in rat pancreatic acinar cells (Muallem *et al.*, 1989) and bovine aortic endothelial cells (BAEC) (Thuringer *et al.*, 1991) have shown that alkalinization of the extracellular buffer stimulates agonist-induced Ca^{2+} influx. And sixth, the agonist-activated influx pathway, like all known Ca^{2+} channels (Hagiwara & Byerly, 1981), is permeable to Ba^{2+} as well as Ca^{2+} (Schilling *et al.*, 1989; Kwan & Putney, 1990).

Recent studies in neutrophils (Smith *et al.*, 1990) and endothelial cells (Schilling *et al.*, 1991) suggest that inhibition of phospholipase C attenuates both the agonist-stimulated release of Ca^{2+} from internal stores and the influx of Ca^{2+} from the

Abbreviations used: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; BAECs, bovine aortic endothelial cells; BHQ, 2,5-di-t-butylhydroquinone; CPAEs, calf pulmonary artery endothelial cells; DMEM, Dulbecco's Modified Eagle's Medium; fura-2/AM, fura-2 acetoxymethyl ester; HBS, Hepes-buffered saline; SKF 96365, 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride; pH_o, extracellular pH.

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extracellular space. Since both phases of the response appear to depend on phosphoinositide hydrolysis, stimulation of Ca^{2+} influx may involve either a direct activation of a surface membrane channel by Ins(1,4,5)P_3 and/or Ins(1,3,4,5)P_4, or may simply reflect the ability of these second messengers to deplete the internal stores of Ca^{2+}, as suggested by the capacitative model proposed by Putney (1986, 1987, 1990). Several agents, specifically 2,5-di-t-butylhydroquinone (BHQ), thapsigargin and cyclopiazonic acid, have proved useful in testing the latter hypothesis. These compounds are selective inhibitors of the Ca^{2+}-ATPase of the endoplasmic and/or sarcoplasmic reticulum, and appear to deplete internal cellular stores of Ca^{2+} without apparent increases in phosphoinositide hydrolysis (Goeger *et al.*, 1988; Goeger & Riley, 1989; Seidler *et al.*, 1989; Takemura *et al.*, 1989; Foder *et al.*, 1989; Kass *et al.*, 1989, 1990; Oldershaw & Taylor, 1990). A number of studies have now shown that these compounds will stimulate Ca^{2+} influx in some non-excitable cells (Takemura *et al.*, 1989; Foder *et al.*, 1989; Kwan *et al.*, 1990; Mason *et al.*, 1991), but not in others (Kass *et al.*, 1989; Lückhoff & Busse, 1990*b*; Llopis *et al.*, 1991). Furthermore, identity between the influx pathway activated by depletion of internal Ca^{2+} stores and that activated by agonists has not been convincingly demonstrated. Thus the purpose of the present study was to compare and contrast the Ca^{2+} influx activated by inhibition of the Ca^{2+}-ATPase with that stimulated by bradykinin in vascular endothelial cells. The results suggest that (1) in the absence of phosphoinositide hydrolysis, depletion of the agonist-sensitive internal Ca^{2+} store in endothelial cells is sufficient for activation of Ca^{2+} influx, and (2) the agonist-activated Ca^{2+}-influx pathway and the influx pathway activated by depletion of the Ins(1,4,5)P_3-sensitive internal store are indistinguishable.

MATERIALS AND METHODS

Solutions and reagents

Unless otherwise indicated, Hepes-buffered saline (HBS) contained the following: 140 mM-NaCl, 5.4 mM-KCl, 1 mM-$MgCl_2$, 1.8 mM-$CaCl_2$, 10 mM-D-glucose, 0.1% BSA and 15 mM-Na-Hepes, pH 7.4 at 37 °C. Zero-Ca^{2+}/EGTA buffer contained 0.3 mM-EGTA and the same salts as HBS but without added $CaCl_2$. Fura-2 acetoxymethyl ester (fura-2/AM) was obtained from Molecular Probes (Eugene, OR, U.S.A.). Bradykinin and ionomycin were obtained from Calbiochem (San Diego, CA, U.S.A.). BHQ was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) and was used from a 10 mM stock solution (in ethanol). Thapsigargin and cyclopiazonic acid were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and were used from 200 μM and 10 mM stock solution in ethanol respectively. SKF 96365 (hydrochloride salt) was generously given by Dr. Janet Merritt (SmithKline Beecham Pharmaceuticals, U.K.). In some experiments, NaCl in the buffer was iso-osmotically replaced by KCl. In the experiments with alkaline extracellular solution, the buffer contained 10 mM-Tris base and 10 mM-Hepes, with pH adjusted to 7.4, 8.0, 8.5 or 9.0 at 37 °C with NaOH. All other chemicals and salts were of reagent grade.

Culture of vascular endothelial cells

Calf pulmonary artery endothelial cells (CPAEs) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% (v/v) fetal-bovine serum, 100 μg of streptomycin and penicillin/ml and 2 mM-glutamine as previously described (Elliott & Schilling, 1990). BAEC were isolated and cultured as previously described (Colden-Stanfield *et al.*, 1987; Schilling *et al.*, 1988). Experiments were performed with cells in passages 9–18 and at 1–3 days post-confluency.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured by using the fluorescent indicator fura-2, as previously described (Schilling *et al.*, 1989). Briefly, the cells were enzymically dispersed, washed and resuspended in HBS containing 20 μM-fura-2/AM. After incubation for 30 min at 37 °C, the cell suspension was diluted > 10-fold and incubated for an additional 30 min, washed, and resuspended. Samples from this final suspension were centrifuged (5 min at 100 *g*) and washed twice immediately before measurement of fluorescence with an SLM 8000 spectrophotofluorimeter. Excitation wavelength alternated every 0.5 s between 340 and 380 nm, and emission was monitored at 510 nm. All measurements were corrected for autofluorescence by using unloaded cells. Calibration of the fura-2 associated with the cells was accomplished by using Triton lysis in the presence of saturating bivalent-cation concentration, followed by addition of EGTA. $[Ca^{2+}]_i$ was calculated from the equation of Grynkiewicz *et al.* (1985). BHQ, thapsigargin and cyclopiazonic acid had no effect on fura-2 fluorescence itself, or on autofluorescence of unloaded cells when examined at concentrations employed in this study.

Measurement of $^{45}Ca^{2+}$ uptake into endothelial-cell monolayers

Uptake of $^{45}Ca^{2+}$ into cell monolayers was performed as previously reported (Schilling *et al.*, 1989; Elliott & Schilling, 1991). Briefly, uptake was initiated by addition of a small portion of a solution containing $^{45}Ca^{2+}$ (final concn. 10–25 μCi/ml) alone or $^{45}Ca^{2+}$ plus bradykinin to confluent monolayers in 35 mm dishes containing 2 ml of culture medium at 37 °C. Uptake was terminated by rapid aspiration of the reaction medium, followed by washing of the monolayer (three times) with ice-cold HBS containing 0.2 mM-$LaCl_3$. Radioactivity was determined in NaOH digests of the monolayers.

Measurement of $^{45}Ca^{2+}$ efflux from endothelial-cell monolayers

Efflux of $^{45}Ca^{2+}$ was measured as previously described (Elliott & Schilling, 1991). Briefly, cells were equilibrated with radioisotope (10–25 μCi/ml) for 15–18 h. The medium was aspirated from the culture dish, and the monolayer was immediately washed three times with HBS containing 140 mM-NaCl, 5.4 mM-KCl, 1 mM-$MgCl_2$, 1.8 mM-$CaCl_2$, 10 mM-D-glucose and 10 mM-Na-Hepes, pH 7.4, at 37 °C. After the final aspiration, 3 ml of HBS was added to the dish, and the cells were allowed to equilibrate for 5 min, at which time duplicate 100 μl samples were withdrawn (zero time for all experiments) before addition of test agents. At each subsequent time point, duplicate samples were withdrawn and placed in scintillation vials. Radioactivity was determined by standard liquid-scintillation technique. All values are expressed as the percentage of radioisotope remaining associated with the cell monolayer relative to the value at the end of the 5 min equilibration period.

Measurement of phosphoinositide hydrolysis

The activity of phosphoinositol-specific phospholipase C was determined in endothelial-cell monolayers as previously described (Liao *et al.*, 1990). Briefly, cells were seeded in 35 mm culture dishes and incubated with *myo*-[^{3}H]inositol (10 μCi/ml) for 48 h. Labelled cells were washed and allowed to equilibrate at 37 °C with serum-free DMEM for 45–60 min. The medium was removed, the cells were rinsed twice with phosphate-buffered saline (GIBCO) supplemented with 5.5 mM-glucose, 0.5 mM-$CaCl_2$ and 0.5 mM-$MgCl_2$, and incubated with 2 ml of phosphate-buffered saline for 20 min at 37 °C. After incubation with LiCl (10 mM) for 10 min, timed additions of the test compounds or vehicle

were made. The reactions were terminated at various times by addition of ice-cold $HClO_4$. The free [^{3}H]inositol phosphates accumulated during the incubation were extracted, separated on Dowex AG 1-X8 (100–200 mesh) columns and quantified by liquid-scintillation counting in Aquasol-2 cocktail (New England Nuclear).

RESULTS

Effect of BHQ on $[Ca^{2+}]_i$

The effect of BHQ was compared with the effect of bradykinin on $[Ca^{2+}]_i$ (Fig. 1). Bradykinin produced the normal biphasic response. In contrast, BHQ produced a small step-like increase in $[Ca^{2+}]_i$ at low concentration (0.5 and 1 μM) and a small increase followed by a slowly rising phase at a concentration of 10 μM. At the higher BHQ concentration, $[Ca^{2+}]_i$ eventually increased to the plateau level seen with bradykinin. Similar results were obtained upon application of BHQ to fura-2-loaded BAECs, although the time course of $[Ca^{2+}]_i$ change on addition of BHQ was somewhat slower in BAECs compared with CPAEs (see Fig. 9).

To determine the relative contribution of internal store Ca^{2+} and extracellular Ca^{2+} to the increase seen with BHQ, the response was examined in zero-Ca^{2+}/EGTA buffer (Fig. 2). The increase in $[Ca^{2+}]_i$ observed on addition of BHQ was attenuated in the absence of extracellular Ca^{2+}, and the response was not sustained, but rather slowly declined with time. This result suggests that the source of Ca^{2+} involved in the BHQ response is from both inside and outside the cell. As shown in Fig. 3, the intracellular source of Ca^{2+} appears to be the bradykinin-sensitive compartment. Addition of bradykinin at various times after BHQ led to a progressive decrease in the $[Ca^{2+}]_i$ response. Similar results were obtained with two additional inhibitors of Ca^{2+}-ATPase, thapsigargin and cyclopiazonic acid (Figs. 3*b* and 3*c*). Both compounds increased $[Ca^{2+}]_i$ and the subsequent response of the cells to bradykinin progressively decreased with time after the addition of these compounds.

It is interesting that the sustained level of $[Ca^{2+}]_i$ after bradykinin addition seems to be related to the level of Ca^{2+} that remains associated with the internal store. BHQ appears to deplete more rapidly the internal store (as judged by the peak of the response after addition of bradykinin after BHQ), and the plateau $[Ca^{2+}]_i$ is very similar for each bradykinin addition (Fig. 3*a*). However, thapsigargin and cyclopiazonic acid seem to deplete the internal store more slowly, and a sustained elevated phase can clearly be observed when bradykinin is added after only a short interval (Figs. 3*b* and 3*c*).

Further identification of the intracellular store involved in the response to BHQ was accomplished by examining the effect of ionomycin and bradykinin added at various times after the application of BHQ to cells bathed in zero-Ca^{2+}/EGTA buffer. In contrast with the $[Ca^{2+}]_i$ response seen after bradykinin

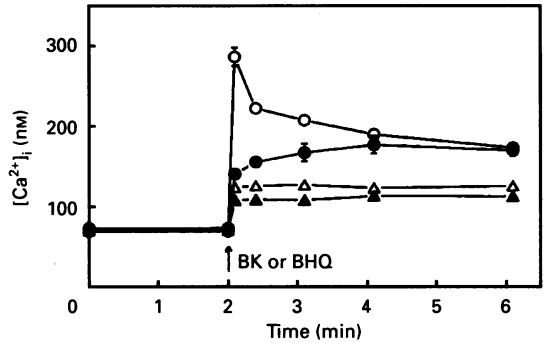

Fig. 1. Comparison of bradykinin- and BHQ-induced changes in $[Ca^{2+}]_i$ of CPAEs

At the time indicated by the arrow, bradykinin (BK; 50 nM; ○), or 0.5 μM- (▲), 1.0 μM- (△) or 10 μM- (●) BHQ was added to fura-2-loaded cells suspended in normal HBS. Each value represents the mean ± S.E.M. ($n = 4$) of selected time points from the fluorescence recordings. Where not shown, the S.E.M. was smaller than the size of the symbol used.

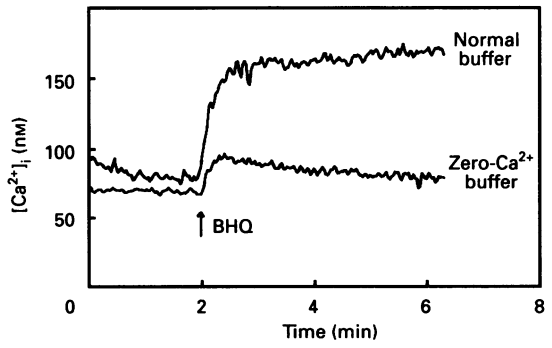

Fig. 2. Effect of BHQ on $[Ca^{2+}]_i$ measured in the absence of extracellular Ca^{2+}

$[Ca^{2+}]_i$ was determined in cells incubated either in normal HBS (upper trace) or in zero-Ca^{2+}/EGTA buffer (lower trace). BHQ (10 μM) was added to the cells at the time indicated by the arrow.

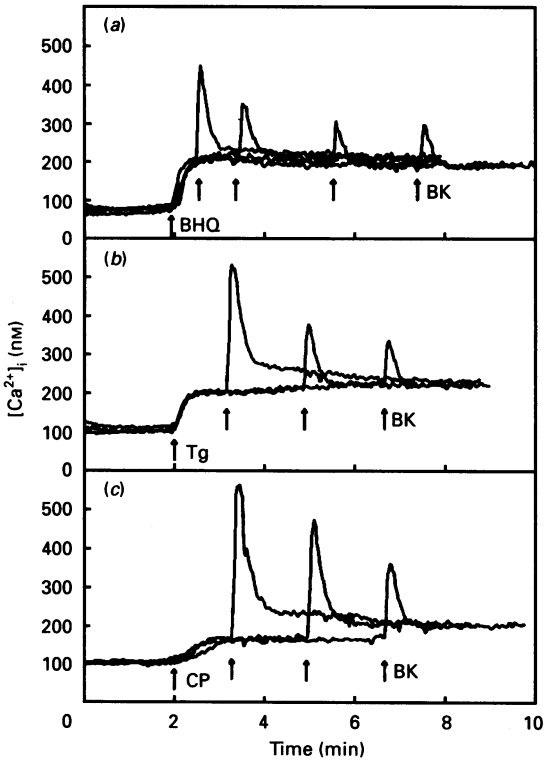

Fig. 3. Time course of internal store depletion produced by BHQ

$[Ca^{2+}]_i$ was determined in CPAEs incubated in normal HBS. In (*a*), several traces are superimposed. In each trace, BHQ (10 μM) was added at 2 min, followed by addition of bradykinin (BK; 50 nM) at the times indicated by the arrows. (*b*) Same as in (*a*), but with thapsigargin (Tg; 200 nM) added at 2 min. (*c*) Same as in (*a*), but with cyclopiazonic acid (CP; 10 μM) added at 2 min. The results shown are representative of at least three experiments.

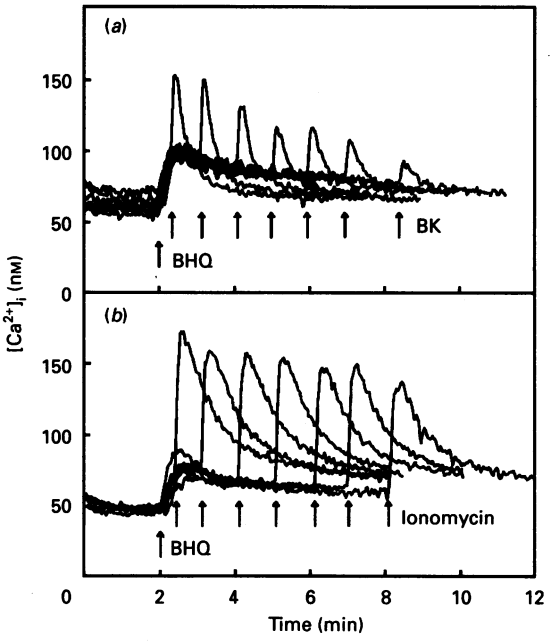

Fig. 4. Time course of internal store depletion produced by BHQ measured in zero-Ca^{2+}/EGTA buffer

$[Ca^{2+}]_i$ was determined in CPAEs incubated in zero-Ca^{2+}/EGTA buffer. The Figure shows several traces superimposed in each panel. In each trace, BHQ (10 μM) was added at 2 min, followed by addition of either bradykinin (BK; 50 nM; *a*) or ionomycin (1 μM; *b*) at the times indicated by the arrows. The results shown are representative of at least three experiments.

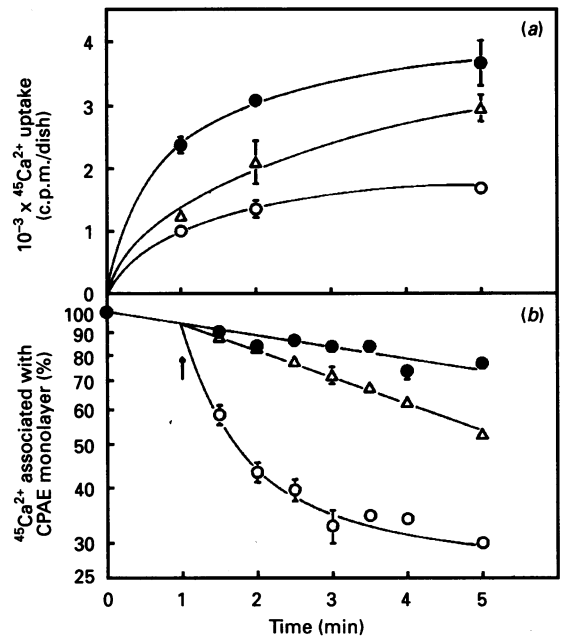

Fig. 5. Comparison of bradykinin- and BHQ-induced increase in $^{45}Ca^{2+}$ uptake and efflux from CPAE monolayers

(*a*) Uptake of $^{45}Ca^{2+}$, determined as described in the Materials and methods section, was initiated by addition of a small amount of solution containing $^{45}Ca^{2+}$ (○), $^{45}Ca^{2+}$ and bradykinin (75 nM; ●), or $^{45}Ca^{2+}$ and BHQ (15 μM; △). Uptake was terminated at the indicated times by rapid aspiration of the uptake buffer and washing with ice-cold HBS containing 0.2 mM-$LaCl_3$. (*b*) Efflux of $^{45}Ca^{2+}$ was determined in CPAE monolayers as described in the Materials and methods section under basal conditions (●) and after addition of bradykinin (50 nM; ○) or BHQ (10 μM; △) at the time indicated by the arrow. Values represent means ± S.E.M. for 3–4 monolayers. Where not shown, the S.E.M. was smaller than the size of the symbol used.

addition, which progressively declined over 6 min (Fig. 4*a*), the response of the cells to ionomycin decreased little over this time frame (Fig. 4*b*). Similar results (not shown) were obtained with thapsigargin and cyclopiazonic acid. Thus it appears that the bradykinin-sensitive internal Ca^{2+} store is more sensitive to depletion by these compounds. Similar results (not shown) were obtained with BAECs.

Comparison of bradykinin- and BHQ-induced changes in $^{45}Ca^{2+}$ efflux and influx

The results of the fura-2 experiments suggest that BHQ causes the depletion of the bradykinin-sensitive internal store and concomitantly stimulates Ca^{2+} influx from the extracellular space. To test these hypotheses more directly, we compared BHQ-stimulated $^{45}Ca^{2+}$ efflux and influx with that observed with bradykinin (Fig. 5). As previously reported (Schilling *et al.*, 1989; Elliott & Schilling, 1991), addition of bradykinin produced a large increase in both $^{45}Ca^{2+}$ efflux and influx. Likewise, BHQ produced an increase in both the efflux and influx of $^{45}Ca^{2+}$ examined in CPAE monolayers, although the increase seen with BHQ was significantly smaller than that obtained with bradykinin. BHQ (10 μM) had no effect on $^{86}Rb^{+}$ efflux from CPAE monolayers (results not shown), suggesting that BHQ does not cause a generalized increase in plasmalemma ionic permeability.

Comparison of bradykinin- and BHQ-induced changes in phosphoinositide hydrolysis in CPAEs

Bradykinin produced a 2.8 ± 0.2-fold and 3.0 ± 0.2-fold increase in the accumulation of total inositol phosphates measured in CPAEs either 30 s or 5 min after the addition of the agonist, respectively. In contrast, BHQ had no significant effect on phosphoinositide hydrolysis (1.1 ± 0.1-fold and 1.1 ± 0.02-fold increase at 30 s and 5 min respectively). Addition of bradykinin to cell monolayers pre-treated with BHQ for 5 min produced an increase in inositol phosphate accumulation that was not significantly different from control, demonstrating that BHQ has no direct effect on bradykinin-dependent activation of phospholipase C. These results suggest that BHQ-dependent changes in $[Ca^{2+}]_i$ occur via a mechanism that is independent of phosphoinositide hydrolysis.

Characteristics of the agonist-activated Ca^{2+}-influx pathway

As shown above, application of bradykinin to fura-2-loaded endothelial cells suspended in HBS containing 2 mM-KCl (low-K^+ buffer) produced a rapid 4–6-fold increase in $[Ca^{2+}]_i$ over basal values and subsequently declined to a sustained elevated level (Fig. 6). $[(Ca^{2+}]_i$ rapidly decreased to basal values after addition of the inorganic Ca^{2+}-influx blocker La^{3+} (50 μM) during the sustained phase of the response. Application of bradykinin to cells depolarized by suspension in HBS containing 140 mM-KCl (high-K^+ buffer) also produced a rapid 4–6-fold increase in $[Ca^{2+}]_i$ over the basal value; however, the sustained phase of the response observed in low-K^+ buffer was attenuated and subsequent application of La^{3+} had no effect on $[Ca^{2+}]_i$. Application of bradykinin to cells suspended in low-K^+ buffer with La^{3+} present before the agonist produced a response essentially identical with that obtained in high-K^+ buffer (results not shown). These results are consistent with our previous studies (Colden-Stanfield *et al.*, 1987; Schilling *et al.*, 1989; Schilling, 1989) and suggest that bradykinin stimulates the influx of Ca^{2+} through a La^{3+}- and membrane-potential-sensitive mechanism.

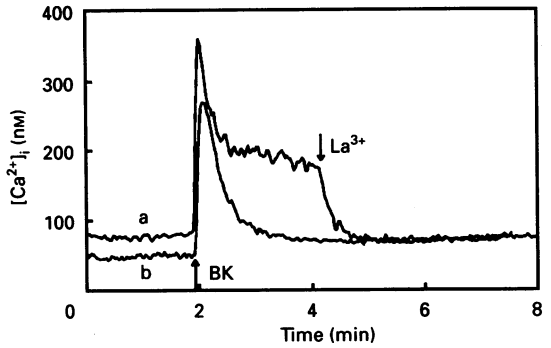

Fig. 6. Effect of La^{3+} and membrane depolarization on bradykinin-induced changes in $[Ca^{2+}]_i$

Fura-2-loaded CPAEs were incubated in HBS containing 2 mM-KCl (trace a) or 140 mM-KCl (trace b). At approx. 2 min, bradykinin (BK; final concn. 50 nM) was added to the cell suspensions and the fluorescence recorded. At the time indicated by the second arrow, $LaCl_3$ (final concn. 50 μM) was added to each.

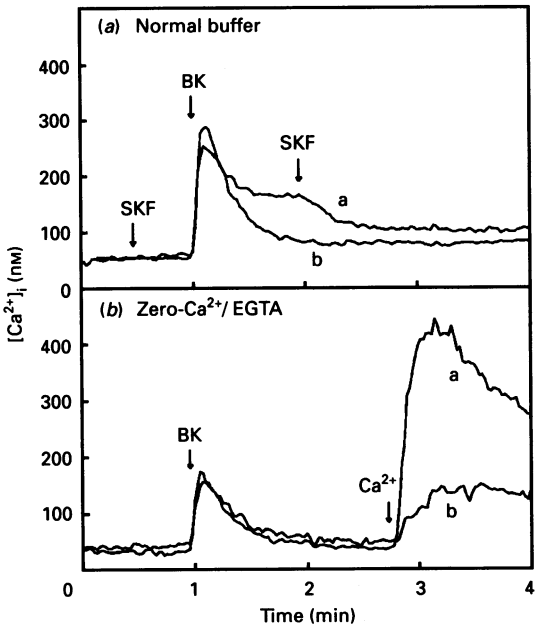

Fig. 7. Effect of SKF 96365 on bradykinin-induced changes in $[Ca^{2+}]_i$

Two traces are superimposed in each panel. (*a*) Bradykinin (BK; 50 nM) and SKF 96365 (50 μM) were added at the times indicated by the arrows to fura-2-loaded BAECs suspended in normal HBS: trace a, SKF 96365 was added after bradykinin; trace b, SKF 96365 was added before bradykinin. (*b*) Bradykinin (50 nM) and Ca^{2+} (2 mM) were added to fura-2-loaded BAECs suspended in zero-Ca^{2+}/EGTA buffer in the absence (trace a) or presence (trace b) of SKF 96365 (50 μM).

Agonist-activated Ca^{2+} influx can also be inhibited by the novel synthetic compound SKF 96365 (Merritt *et al.*, 1990). Application of bradykinin to cells suspended in normal HBS resulted in the typical biphasic response (Fig. 7*a*). Addition of SKF 96365 (50 μM) during the sustained component produced a decrease in $[Ca^{2+}]_i$ toward basal levels, and application of SKF 96365 before bradykinin eliminated the plateau phase in a fashion similar to that with La^{3+}. To determine if SKF 96365 affected bradykinin-induced Ca^{2+} release from internal stores, the response of the cells was examined in zero-Ca^{2+}/EGTA

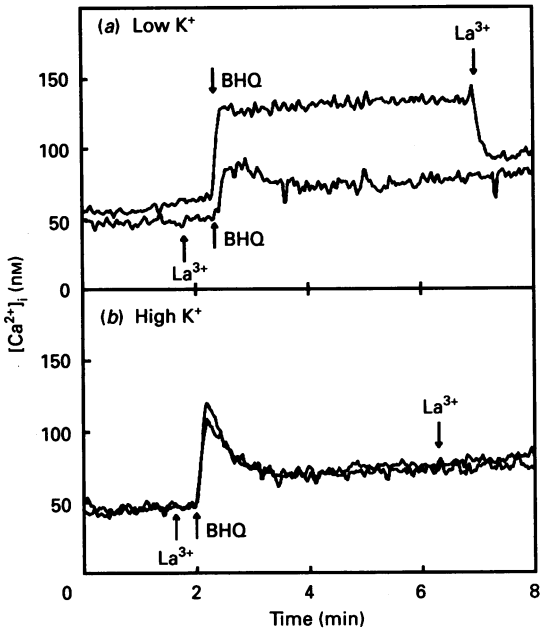

Fig. 8. Effect of La^{3+} and membrane depolarization on BHQ-induced changes in $[Ca^{2+}]_i$

Two traces are superimposed in each panel. (*a*) BHQ (10 μM) and $LaCl_3$ (50 μM) were added at the times indicated by the arrows to fura-2-loaded CPAEs suspended in HBS containing 2 mM-KCl. In the upper trace $LaCl_3$ was added after BHQ, whereas in the lower trace $LaCl_3$ was added before BHQ. (*b*) Same as in (*a*), but with the cells suspended in HBS containing 140 mM-KCl.

buffer (Fig. 7*b*). Under these conditions, addition of bradykinin produced a 3–4-fold increase in $[Ca^{2+}]_i$ indicative of Ca^{2+} release from internal stores. $[Ca^{2+}]_i$ reached a peak within seconds and subsequently returned to basal levels within 1 min after addition of bradykinin. Re-addition of Ca^{2+} to the extracellular buffer after bradykinin resulted in a large increase in $[Ca^{2+}]_i$, indicative of influx via the agonist-activated pathway (Schilling *et al.*, 1988, 1989). In the presence of SKF 96365, the bradykinin-induced increase in $[Ca^{2+}]_i$ was unchanged from control, whereas the rise in $[Ca^{2+}]_i$ seen after re-addition of Ca^{2+} to the extracellular buffer was significantly attenuated. These results demonstrate that SKF 96365 blocks agonist-activated Ca^{2+} influx, but has no apparent effect on bradykinin-induced Ca^{2+} release from internal stores.

Characteristics of the Ca^{2+}-influx pathway activated by depletion of the Ins(1,4,5)P_3-sensitive internal store

The next set of experiments examined the ability of extracellular La^{3+} and membrane depolarization to inhibit BHQ-induced Ca^{2+} influx. As shown in Fig. 8(*a*), addition of BHQ to cells suspended in low-K^+ buffer produced a 2–3-fold increase in $[Ca^{2+}]_i$ that remained elevated over the basal value for several minutes. Application of La^{3+} after BHQ resulted in a decrease in $[Ca^{2+}]_i$ toward basal values. Addition of La^{3+} before BHQ significantly attenuated the response, which reached a peak and subsequently declined. Addition of BHQ to cells suspended in high K^+ produced only a transient increase in $[Ca^{2+}]_i$ and La^{3+} was without effect when added either before or after BHQ (Fig. 8*b*). Thus it appears that the Ca^{2+} influx activated by BHQ is sensitive to La^{3+} and is attenuated by depolarization of the cells. Similar results were obtained when the internal stores were released by either thapsigargin (200 nM) or cyclopiazonic acid (10 μM) (results not shown). Both compounds produced a sustained increase in $[Ca^{2+}]_i$ in low-K^+ buffer that was blocked by La^{3+}. Likewise, the

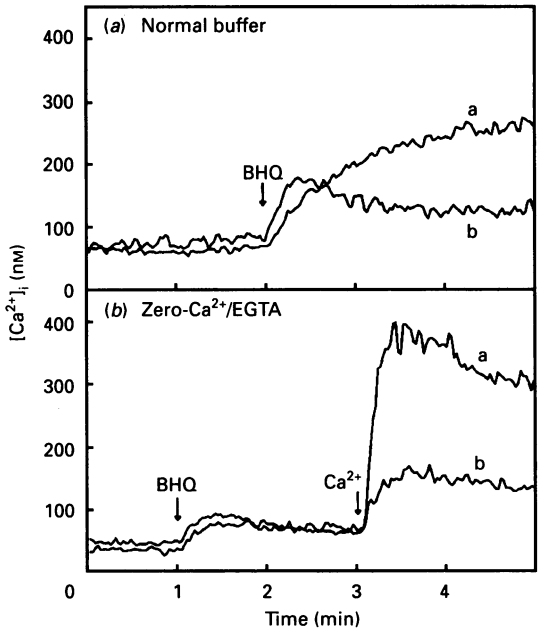

Fig. 9. Effect of SKF 96365 on BHQ-induced changes in $[Ca^{2+}]_i$

Two traces are superimposed in each panel. (*a*) BHQ (10 μM) was added at the time indicated by the arrow to fura-2-loaded BAECs suspended in normal HBS. Trace a was recorded in the absence of SKF 96365, whereas trace b was obtained in the presence of SKF 96365 (50 μM). (*b*) BHQ (10 μM) and Ca^{2+} (2 mM) were added to fura-2-loaded BAECs suspended in zero-Ca^{2+}/EGTA buffer in the absence (trace a) or presence (trace b) of SKF 96365 (50 μM). Note that the time course of $[Ca^{2+}]_i$ change after application of BHQ is slower in BAECs than in CPAEs.

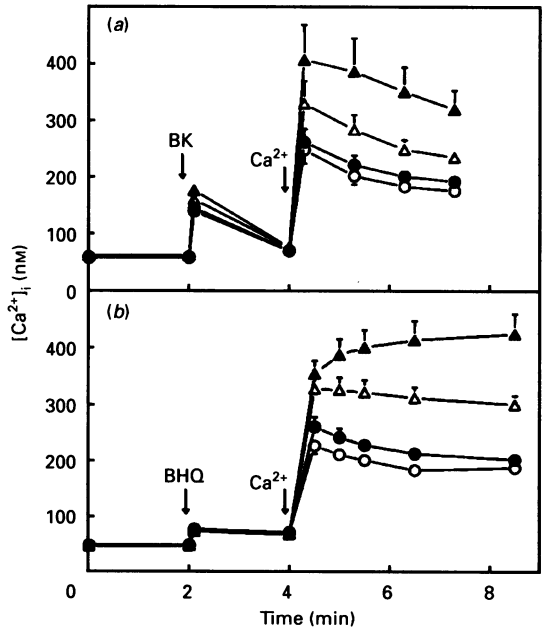

Fig. 10. Effect of alkalinization of the extracellular buffer on agonist- and BHQ-induced Ca^{2+} influx

$[Ca^{2+}]_i$ was monitored in fura-2-loaded CPAEs incubated in zero-Ca^{2+}/EGTA buffer with the pH adjusted to 7.4 (○), 8.0 (●), 8.5 (△) and 9.0 (▲). At the times indicated by the arrows, either bradykinin (BK; 50 nM) followed by Ca^{2+} (1.7 mM) (*a*) or BHQ (50 μM) followed by Ca^{2+} (1.7 mM) (*b*) was added to the cell suspension and the fluorescence recorded. The points represent the mean (±S.E.M.) $[Ca^{2+}]_i$ values at selected time points ($n = 5$ determinations at each pH_o). Where not shown, the S.E.M. was smaller than the size of the symbol used.

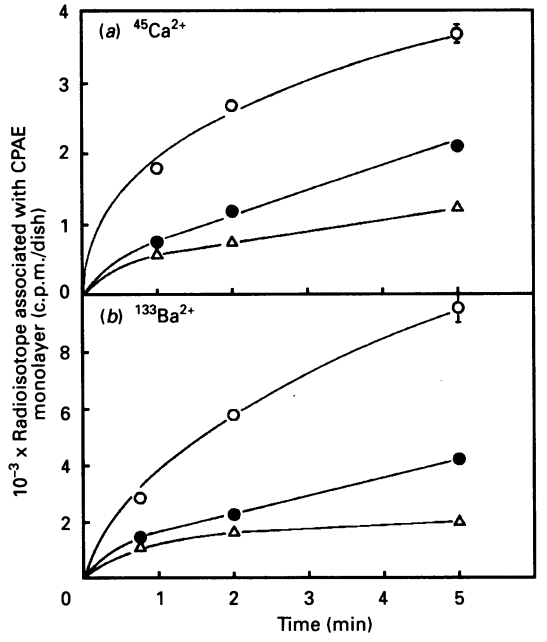

Fig. 11. Comparison of bradykinin- and BHQ-stimulated $^{45}Ca^{2+}$ and $^{133}Ba^{2+}$ uptake

(*a*) Uptake of $^{45}Ca^{2+}$ was initiated by addition of a small amount of solution containing $^{45}Ca^{2+}$ (△), $^{45}Ca^{2+}$ and bradykinin (75 nM; ○), or $^{45}Ca^{2+}$ and BHQ (15 μM; ●). (*b*) Uptake of $^{133}Ba^{2+}$ was initiated by addition of a small portion of solution containing $^{133}Ba^{2+}$ (△), $^{133}Ba^{2+}$ and bradykinin (50 nM; ○), or $^{133}Ba^{2+}$ and BHQ (10 μM; ●). Uptake was terminated at the indicated times by rapid aspiration of the uptake buffer and washing with ice-cold HBS containing 0.2 mM-$LaCl_3$. Each value represents the mean ± S.E.M. from three monolayers. Where not shown, the S.E.M. was smaller than the size of the symbol used.

response of the cells to thapsigargin and cyclopiazonic acid was attenuated in high-K^+ buffer.

BHQ-induced influx of Ca^{2+} was also inhibited by SKF 96365 (Fig. 9). In cells suspended in normal HBS, SKF 96365 blocked the sustained increase seen on application of BHQ. In cells suspended in zero-Ca^{2+}/EGTA buffer, SKF 96365 had no effect on the initial elevation produced by BHQ; however, the response to subsequent re-addition of Ca^{2+} to the extracellular buffer was significantly decreased. Additionally, SKF 96365 produced an inhibition of the sustained component of the response to BHQ in low-K^+ buffer, but had no effect on the transient response seen on application of BHQ to cells suspended in high-K^+ buffer. SKF 96365 also inhibited the sustained increase in $[Ca^{2+}]_i$ produced by thapsigargin (200 nM) and cyclopiazonic acid (10 μM) (results not shown).

Effect of organic inhibitors of voltage-gated Ca^{2+} channels on BHQ-induced changes in $[Ca^{2+}]_i$

Previous studies have shown that agonist-activated Ca^{2+} influx is insensitive to the organic Ca^{2+}-channel antagonists (Colden-Stanfield *et al.*, 1987). The increase in $[Ca^{2+}]_i$ observed on addition of BHQ was unaffected by either nitrendipine (1 μM) or BAY K 8644 (1 μM) added either before or after BHQ (results not shown).

Effect of extracellular pH (pH_o) on bradykinin- and BHQ-induced changes in $[Ca^{2+}]_i$

The effect of alkalinization of the extracellular buffer on the $[Ca^{2+}]_i$ response to bradykinin was examined in cells incubated in zero-Ca^{2+}/EGTA solutions with pH in the range 7.4–9.0 (Fig.

10*a*). Application of bradykinin under these conditions produced a 2–3-fold increase in $[Ca^{2+}]_i$ that decreased to basal values within 1–2 min. Neither the time course nor the magnitude of the $[Ca^{2+}]_i$ response to bradykinin was significantly affected by variation in pH_o. Re-addition of Ca^{2+} to the extracellular solution after bradykinin produced a rapid increase in $[Ca^{2+}]_i$ which declined slowly with time. The magnitude of the $[Ca^{2+}]_i$ change observed at pH_o 8.0 was not significantly different from that seen at pH_o 7.4. However, increasing pH_o to 8.5 and 9.0 produced a graded increase in the magnitude of the $[Ca^{2+}]_i$ peak without change in the subsequent time course of $[Ca^{2+}]_i$ decline.

The change in $[Ca^{2+}]_i$ on addition of BHQ to cells incubated in zero-Ca^{2+}/EGTA buffer was unaffected by alkalinization of the extracellular solution (Fig. 10*b*). However, re-addition of Ca^{2+} after BHQ again produced an increase in $[Ca^{2+}]_i$ which exhibited a sensitivity to pH_o similar to that seen for bradykinin. However, after the peak response, the rate of decline in $[Ca^{2+}]_i$ was slowed at pH_o 8.5, and $[Ca^{2+}]_i$ continued to increase slowly to a new sustained level when challenged with Ca^{2+} after BHQ at pH_o 9.0.

Comparison of bradykinin- and BHQ-stimulated $^{45}Ca^{2+}$ and $^{133}Ba^{2+}$ influx

Previous studies using fura-2 have suggested that the bradykinin-stimulated influx pathway is permeable to both Ca^{2+} and Ba^{2+}. To determine if the influx pathway activated by depletion of the internal store also allows Ba^{2+} influx, uptake of $^{45}Ca^{2+}$ and $^{133}Ba^{2+}$ was examined (Fig. 11). Bradykinin produced a large increase in both $^{45}Ca^{2+}$ and $^{133}Ba^{2+}$ influx. Likewise, BHQ stimulated influx of both radioisotopes, although to a lesser extent compared with the bradykinin-stimulated component. However, it is clear that BHQ stimulated uptake of both radioisotopes to the same extent, suggesting that the BHQ-sensitive pathway is permeable to both Ba^{2+} and Ca^{2+}.

DISCUSSION

Although it is well established that agonist agents activate both the release of Ca^{2+} from internal stores and the influx of Ca^{2+} from the extracellular space, the molecular mechanisms associated with receptor-mediated Ca^{2+} influx remain unknown. Several studies in non-excitable cells have suggested that influx is coupled to the level of Ca^{2+} within the internal store and that the influx pathway remains activated after removal of the agonist, until the internal store is again replete with Ca^{2+} (Merritt & Rink, 1987; Pandol *et al.*, 1987; Muallem *et al.*, 1988; Jacob, 1990; Mertz *et al.*, 1990). These results suggest that sustained phosphoinositide hydrolysis is not necessary for the continued activation of Ca^{2+} influx. However, it is possible that transient elevation of inositol phosphates, $[Ca^{2+}]_i$, or some other second messenger may lead to a prolonged activation of Ca^{2+} influx, or alternatively that the influx pathway activated by agonist is different from that responsible for refilling of the internal stores after termination of the agonist–receptor interaction. In this regard, Jacob (1990) showed that Mn^{2+} entry into human umbilical-vein endothelial cells after agonist stimulation occurs via a pathway that is controlled by the degree of fullness of the internal store, and that this pathway is, at least in part, the same pathway by which Ca^{2+} refills the internal store.

Previous studies in parotid and lacrimal acinar cells (Takemura *et al.*, 1989; Kwan *et al.*, 1990), neutrophils (Foder *et al.*, 1989), and lymphocytes (Mason *et al.*, 1991) have shown that the tumour promoter thapsigargin, a selective inhibitor of the Ca^{2+}-ATPase of the endoplasmic reticulum, elevates $[Ca^{2+}]_i$ and stimulates the influx of Ca^{2+}. Although identity between the influx pathway activated by depletion of the internal store and that activated by agonist agents has not been clearly demonstrated, it appears that, in the absence of receptor stimulation and phosphoinositide hydrolysis, Ca^{2+} influx can be stimulated by depletion of the internal stores. In hepatocytes. Moore *et al.* (1987) have shown that BHQ selectively inhibits the Ca^{2+}-ATPase of the intracellular store. However, in contrast with results obtained in other cell types with thapsigargin, elevation of $[Ca^{2+}]_i$ by BHQ in hepatocytes results solely from the release of Ca^{2+} from internal stores without concomitant stimulation of Ca^{2+} influx (Kass *et al.*, 1989; Llopis *et al.*, 1991). A similar result was obtained by Lückhoff & Busse (1990*b*) in pig aortic endothelial cells. However, these studies relied, in part, on Mn^{2+} quenching of intracellular fura-2 for assessment of Ca^{2+} influx. It is possible that Ca^{2+} entry may occur via a pathway that is not permeable to Mn^{2+} (Llopis *et al.*, 1991). Alternatively, these results suggest that the release of Ca^{2+} from the internal store is not sufficient for stimulation of influx, and would be consistent with the requirement for receptor stimulation and/or the opening of Ins(1,4,5)P_3-dependent Ca^{2+} channels of the endoplasmic reticulum. Thus the mechanism of agonist-stimulated Ca^{2+} influx may differ with cell type (Llopis *et al.*, 1991).

Is depletion of the internal Ca^{2+} store sufficient for activation of Ca^{2+} influx in endothelial cells?

The results of the present study suggest that agonist-induced depletion of the Ins(1,4,5)P_3-sensitive internal Ca^{2+} store in CPAEs and BAECs is sufficient for activation of Ca^{2+} influx from the extracellular space. BHQ produced a sustained increase in $[Ca^{2+}]_i$ that was dose-dependent and attenuated in the absence of extracellular Ca^{2+}. BHQ stimulated $^{45}Ca^{2+}$ efflux from intact cells, but had no effect on phosphoinositide hydrolysis or on $^{86}Rb^+$ efflux from intact cells. Similar results on $[Ca^{2+}]_i$ were obtained with two other putative inhibitors of the endoplasmic reticulum Ca^{2+}-ATPase, i.e. thapsigargin and cyclopiazonic acid. Thus it appears that the initial rise in $[Ca^{2+}]_i$ produced by BHQ reflects inhibition of Ca^{2+} uptake via the endoplasmic-reticulum Ca^{2+} pump and concomitant stimulation of Ca^{2+} influx, a conclusion directly supported by the finding that BHQ stimulates $^{45}Ca^{2+}$ uptake into intact cells.

It is noteworthy that there appears to be tight coupling between the level of Ca^{2+} within the internal store and the magnitude of the Ca^{2+} influx. As illustrated in Fig. 4, shortly after inhibition of the Ca^{2+}-ATPase, application of bradykinin produces a large $[Ca^{2+}]_i$ transient that is followed by a sustained elevated phase. However, after longer incubations with the Ca^{2+}-pump inhibitors, the peak of the $[Ca^{2+}]_i$ transient produced by bradykinin decreases, and the plateau is not elevated over that seen with the inhibitors alone. These results suggest that, as the Ins(1,4,5)P_3-sensitive internal store becomes depleted of Ca^{2+}, subsequent addition of agonist elicits a progressively smaller influx component. A similar result was taken by Takemura *et al.* (1989) as evidence for identity between the agonist-activated influx pathway and the pathway activated by depletion of the internal stores (see below).

Is the Ins(1,4,5)P_3-sensitive internal store coupled to the plasmalemma influx pathway?

A question that has remained unresolved is whether depletion of a specific compartment within the cell is necessary and/or sufficient for activation of influx. In rat brain microsomes, rat hepatocytes, and lacrimal and parotid acinar cells, the Ins(1,4,5)P_3-sensitive pool appears to be a subset of the thapsigargin-sensitive pool (Takemura *et al.*, 1989; Thastrup *et al.*, 1990; Kwan *et al.*, 1990; Verma *et al.*, 1990; Llopis *et al.*, 1991). Likewise, in permeabilized rat hepatocytes, the Ins(1,4,5)P_3-sensitive store appears to be a subset of the BHQ-

sensitive pool, and BHQ has a slight selectivity for the Ca^{2+}-ATPase/pump of the Ins(1,4,5)P_3-sensitive store (Oldershaw & Taylor, 1990). A similar result was obtained in the present study. Application of BHQ, thapsigargin or cyclopiazonic acid resulted in a time-dependent attenuation of the response to subsequent application of agonist. This result suggests that inhibition of the Ca^{2+} pump does not cause an instantaneous depletion of the internal store, but that depletion occurs in a time-dependent fashion. Thus the initial increase in $[Ca^{2+}]_i$ observed after application of the Ca^{2+}-ATPase/pump inhibitors reflects establishment of a new steady state with respect to influx and efflux of Ca^{2+} from the cytosolic compartment, as expected for a pump-leak model. The rate of decay of the bradykinin response after pump inhibition reflects the rate of passive efflux of Ca^{2+} from internal storage sites. In contrast, the response to addition of ionomycin after inhibition of the Ca^{2+}-ATPase/pump in the absence of extracellular Ca^{2+} decreased very little. The magnitude of this decreased ionomycin response is consistent with loss of the bradykinin-sensitive component. These results strongly support the hypothesis that the BHQ-sensitive compartment, and hence the compartment that is linked to activation of the influx pathway, is the bradykinin-sensitive internal store, i.e. the Ins(1,4,5)P_3-sensitive store. The identity of the Ca^{2+} compartment that appears to be relatively insensitive to depletion by these agents is unknown. Perhaps the Ca^{2+}-ATPase of this compartment is resistant to inhibition by these compounds. In membrane vesicles from human platelets, two distinct Ca^{2+}-pump isoforms exhibiting either high or low sensitivity to thapsigargin have been identified (Papp *et al.*, 1991). Alternatively, it is possible that some other Ca^{2+}-flux pathway is responsible for loading of this store. In this regard, Dehlinger-Kremer *et al.* (1991) suggest that Ca^{2+} uptake into different intracellular compartments of pancreatic acinar cells occurs either via a Ca^{2+}/H^+ exchanger or via a Ca^{2+}-ATPase/pump mechanism. Furthermore, these sub-compartments may be differentially sensitive to Ins(1,4,5)P_3 and caffeine. The contribution of each of these compartments to short- and long-term regulation of Ca^{2+} homoeostasis in the vascular endothelial cell is not clear at present.

Is the influx pathway activated by receptor agonists the same as that activated by depletion of the Ins(1,4,5)P_3-sensitive internal store?

Because neither pathway has been identified by electrophysiological techniques, we have taken an indirect approach and compared several characteristic features of bradykinin-induced Ca^{2+} influx with that observed after depletion of the internal stores by BHQ, thapsigargin and cyclopiazonic acid. Six lines of evidence suggest that these pathways are indistinguishable. First, both the agonist-activated influx pathway and that stimulated by BHQ, thapsigargin or cyclopiazonic acid are inhibited by extracellular La^{3+}. Although La^{3+} is considered a rather non-specific Ca^{2+}-influx blocker, inhibiting most known Ca^{2+} channels and carriers, the finding that La^{3+} blocks the sustained phase of the response to BHQ, thapsigargin and cyclopiazonic acid is supportive evidence that these compounds indeed activate influx. Second, depolarization of the cell by incubation in high-K^+ buffer both eliminates the sustained $[Ca^{2+}]_i$ component of the bradykinin-stimulated response and significantly attenuates the sustained component seen with all three of the Ca^{2+}-ATPase/pump inhibitors. Previous studies have shown that cultured vascular endothelial cells have a resting membrane potential between -55 and -70 mV that is maintained by an inwardly rectifying K^+ channel (Colden-Stanfield *et al.*, 1987). Additionally, it has been shown that these cells can be depolarized by increasing extracellular K^+ (Schilling, 1989). Thus the finding of the present study that depolarization attenuates the influx of Ca^{2+} induced by BHQ, thapsigargin and cyclopiazonic acid, without affecting the release of Ca^{2+} from internal stores, suggests that these agents activate Ca^{2+} influx via an electrogenic mechanism. These results would be consistent with the hypothesis that Ca^{2+} entry occurs via a passive permeability pathway in a fashion similar to the bradykinin-activated pathway.

Third, SKF 96365 inhibited the sustained $[Ca^{2+}]_i$ response to bradykinin and to BHQ, thapsigargin and cyclopiazonic acid, again without affecting the ability of these agents to deplete Ca^{2+} from the internal stores. SKF 96365 has recently been shown to inhibit ADP-, thrombin- and U46619 (a thromboxane mimetic)-induced Ca^{2+} entry into platelets, as well as the Ca^{2+} influx produced by *N*-formylmethionyl-leucyl-phenylalanine in neutrophils, and the histamine-induced Ca^{2+} influx in human umbilical-vein endothelial cells (Merritt *et al.*, 1990). Fourth, like the agonist-activated Ca^{2+} influx pathway, the BHQ-induced $[Ca^{2+}]_i$ response was insensitive to the organic Ca^{2+}-channel modulator drugs nitrendipine and BAY K 8644.

Fifth, both the agonist- and the BHQ-activated Ca^{2+} flux are increased by alkalinization of the extracellular buffer. Recent studies in rat pancreatic acinar cells (Muallem *et al.*, 1989) and BAECs (Thuringer *et al.*, 1991) have shown that agonist-induced increases in Ca^{2+} influx are enhanced at alkaline pH_o. This increase in Ca^{2+} influx is not related to an alteration in intracellular pH or to inhibition of plasmalemmal Ca^{2+}-ATPase/pump activity, but may represent a direct modulation of the influx pathway by external H^+ (Muallem *et al.*, 1989). And sixth, both the bradykinin-stimulated influx pathway and that activated by BHQ allow both Ca^{2+} and Ba^{2+} to enter the cell. It is well established that all known Ca^{2+} channels are permeable to Ba^{2+} (Hagiwara & Byerly, 1981), whereas Ba^{2+} is a poor substrate for carriers/pumps which normally transport Ca^{2+} (Vanderkoor & Martonosi, 1971; Steiger *et al.*, 1978; Shine *et al.*, 1978).

Although each of these findings when examined separately cannot eliminate the possibility that two separate and independent Ca^{2+}-influx pathways exist, the similarities between the agonist-activated pathway and that activated by BHQ, thapsigargin and cyclopiazonic acid are striking. Taken together, these results strongly suggest that depletion of the Ins(1,4,5)P_3-sensitive internal Ca^{2+} stores activates the agonist-sensitive Ca^{2+} influx pathway in cultured vascular endothelial cells. One note of caution is that although BHQ, thapsigargin and cyclopiazonic acid appear to be selective inhibitors of the Ca^{2+}-ATPase/pump of the internal store (Goeger *et al.*, 1988; Goeger & Riley, 1989; Seidler *et al.*, 1989; Takemura *et al.*, 1989; Foder *et al.*, 1989; Kass *et al.*, 1989, 1990; Oldershaw & Taylor, 1990) and there are no apparent structural similarities between BHQ (a hydroquinone), thapsigargin (a sesquiterpene lactone; Thastrup *et al.*, 1990) and cyclopiazonic acid (an indoletetramic acid; Goeger *et al.*, 1988; Goeger & Riley, 1989), the experiments of the present study do not eliminate the possibility that these compounds have a direct stimulatory effect on the agonist-activated Ca^{2+}-influx pathway. This seems unlikely however, for the following reasons. First, the activation of Ca^{2+} flux appears to be tightly coupled to depletion of the internal Ca^{2+} stores. Thus, after addition of BHQ, thapsigargin or cyclopiazonic acid, the ability of bradykinin to stimulate Ca^{2+} influx was directly related to the Ca^{2+} load within the internal store. Second, in some cells, specifically hepatocytes and pig aortic endothelial cells, BHQ caused an increase in $[Ca^{2+}]_i$, but had no effect on Ca^{2+} influx from the extracellular space (Kass *et al.*, 1989; Lückhoff & Busse, 1990*b*). These cells, however, clearly exhibit Ca^{2+} influx after application of receptor agonists. Thus it seems likely that these compounds activate Ca^{2+} influx via an indirect mechanism

involving inhibition of the Ca^{2+}-ATPase/pump and the release of Ca^{2+} from internal stores.

In summary, receptor stimulation results in activation of phospholipid C and release of Ins(1,4,5)P_3, which opens receptor-operated channels in the endoplasmic reticulum. Release of Ca^{2+} from the internal store activates influx from the extracellular space through some as yet undefined mechanism. Although we cannot eliminate the possibility that Ins(1,4,5)P_3 or some other metabolite of the inositol phosphate pathway contributes to the agonist influx directly, or augments the influx produced by depletion of the internal Ca^{2+} store, it seems unnecessary to explain the results obtained to date. Finally, the actual path taken by Ca^{2+} upon entry from the extracellular space, although recently suggested to be direct (Takemura *et al.*, 1989; Putney, 1990; Kwan & Putney, 1990), may actually traverse the internal store and subsequently exit into the cytoplasm. Recent preliminary studies, which examined bradykinin-stimulated $^{133}Ba^{2+}$ efflux, suggest that there may be a pathway of direct ionic communication between the extracellular space and the lumen of the endoplasmic reticulum (Schilling & Rajan, 1990). Irrespective of the exact mechanism of Ca^{2+} influx, the results of the present study suggest that the pathway activated by depletion of the Ins(1,4,5)P_3-sensitive internal Ca^{2+} store is similar to the agonist-activated influx pathway with respect to sensitivity to blocking agents, membrane depolarization and permeability to Ca^{2+} and Ba^{2+}. These results not only suggest identity between these Ca^{2+}-influx pathways, but may also provide insight into the mechanism by which non-receptor-mediated Ca^{2+} influx can occur under pathophysiological conditions. For example, changes in $[Ca^{2+}]_i$ have been observed under conditions where cellular energy stores are depleted, such as peroxide-induced oxidant stress (Elliott *et al.*, 1989; Elliott & Schilling, 1990, 1991) or in ischaemia/reperfusion-induced cellular injury (Marban *et al.*, 1989; Hayashi *et al.*, 1989; Walker, 1986; Dhalla *et al.*, 1982; Henry *et al.*, 1977). Depletion of internal Ca^{2+} stores under these conditions could represent the primary event associated with changes in plasmalemma Ca^{2+} permeability leading to Ca^{2+} overload and ultimately to cell death.

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