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Mechanism of rise and decay of 2,5-di-*tert*-butylhydroquinone-induced Ca^{2+} signals in Madin Darby canine kidney cells

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Abstract

We examined the effect of 2,5-di-*tert*-butylhydroquinone (BHQ) on intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) measured by fura-2 fluorimetry in Madin Darby canine kidney (MDCK) cells. BHQ increased $[Ca^{2+}]_i$ in a dose-dependent manner with an EC_{50} of 40 μM. The Ca^{2+} signal showed a slow onset, a gradual decay and a sustained plateau in normal Ca^{2+} medium. Depletion of the endoplasmic reticulum Ca^{2+} store by incubation with 0.1 mM BHQ for 6 min abolished the $[Ca^{2+}]_i$ increase evoked by bradykinin or ATP, suggesting that BHQ depleted the inositol 1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} store. Removal of extracellular Ca^{2+} reduced the BHQ response by 50%. The Ca^{2+} signal was initiated by Ca^{2+} release from the internal store, followed by capacitative Ca^{2+} entry which was abolished by 100 μM La^{3+} or 50 μM Gd^{3+} and was partly inhibited by 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1*H*-imidazole hydrochloride (SKF 96365). After depletion of the endoplasmic reticulum Ca^{2+} store, by incubation with another inhibitor of the endoplasmic reticulum Ca^{2+} pump, thapsigargin for 30 min, BHQ did not elevate $[Ca^{2+}]_i$, suggesting that the BHQ-induced Ca^{2+} influx was largely due to capacitative Ca^{2+} entry, and that BHQ released Ca^{2+} from the thapsigargin-sensitive endoplasmic reticulum store. We investigated the mechanism of decay of the BHQ response. Pretreatemt with La^{3+} (or Gd^{3+}) or alkalization of the extracellular medium to pH 8 significantly potentiated the Ca^{2+} signal, whereas pretreatment with carbonylcyanide *m*-chlorophenylhydrazone (CCCP) or oligomycin, or removal of extracellular Na^+, had no effect. Collectively, our results suggest that BHQ increased $[Ca^{2+}]_i$ in MDCK cells by depleting the endoplasmic reticulum Ca^{2+} store followed by capacitative Ca^{2+} entry, with both pathways contributing equally. The decay of the BHQ response is effected by Ca^{2+} efflux via the plasma membrane Ca^{2+} pump, but not by efflux via Na^+/Ca^{2+} exchange or sequestration by the mitochondria.

Keywords: 2,5-Di-*tert*-butylhydroquinone; Ca^{2+} entry, capacitative; MDCK (Madin Darby canine kidney) cell; La^{3+}; Gd^{3+}

1. Introduction

A transient rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), called a '$[Ca^{2+}]_i$ transient', is a pivotal signal for a variety of cellular responses (Clapman, 1995; Berridge, 1997). Upon stimulation, a $[Ca^{2+}]_i$ transient occurs as a result of extracellular Ca^{2+} influx and/or Ca^{2+} release from intracellular stores. One of the major Ca^{2+} sources for the Ca^{2+} signal is the inositol 1,4,5-trisphosphate (IP_3)-sensitive endoplasmic reticulum Ca^{2+} store (Berridge, 1993). Binding of IP_3 to its receptors on the endoplasmic reticulum causes the active release of Ca^{2+} from the endoplasmic reticulum store. This depletion of the endoplasmic reticulum Ca^{2+} store subsequently triggers Ca^{2+} influx from the extracellular medium via a process termed 'capacitative Ca^{2+} entry' (Putney and Bird, 1993).

Studies of Ca^{2+} homeostasis require specific pharmacological tools to modulate Ca^{2+} influx through plasma membrane channels or Ca^{2+} release from intracellular stores. 2,5-Di-*tert*-butylhydroquinone (BHQ), thapsigargin and cyclopiazonic acid are the three most widely used inhibitors of the endoplasmic reticulum Ca^{2+} pump because they are membrane permeable and have rather high selectivity for the pump (Thastrup et al., 1990; Demaurex et al., 1992). In most cells, these drugs inhibit the pump and result in the passive leakage of Ca^{2+} from the IP_3-sensitive Ca^{2+} store leading to an increase in $[Ca^{2+}]_i$ followed by capacitative Ca^{2+} entry.

In Madin Darby canine kidney (MDCK) cells, a renal epithelial cell line, we previously reported that BHQ or

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thapsigargin elicited a marked increase in $[Ca^{2+}]_i$ with a slow onset, a gradual decay, and a sustained plateau, as measured by changes in fura-2 fluorescence (Jan et al., 1998b,c). We also reported that ATP or the phospholipase C inhibitor 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1*H*-pyrrole-2,5-dione (U73122) activated capacitative Ca^{2+} entry, an effect which was abolished by La^{3+} (Jan et al., 1998a,b). Additionally, thapsigargin has been shown to trigger a Ca^{2+} current and a Ca^{2+}-dependent K^+ current (Delles et al., 1995), reflecting activation of capacitative Ca^{2+} entry. This capacitative Ca^{2+} entry is thought to consist of two components with different pharmacological and biophysical properties (Dietl et al., 1996).

BHQ has been reported to inhibit the endoplasmic reticulum Ca^{2+} pump, leading to capacitative Ca^{2+} entry in many cells, such as adrenal chromaffin cells (Robinson et al., 1992) and hepatocytes (Applegate et al., 1997). Besides its inhibition of the Ca^{2+} pump, BHQ has been shown to directly inhibit cyclo-oxygenase in osteoblast-like cells (Leis et al., 1996). However, the BHQ-evoked Ca^{2+} signal in MDCK cells has not been characterized in detail. In particular, it is not known whether BHQ can activate capacitative Ca^{2+} entry. Further, the mechanisms of the rise and decay of the $[Ca^{2+}]_i$ transient need to be elucidated.

In this study, we examine the BHQ-evoked Ca^{2+} signal in fura-2-loaded MDCK cells. We establish the dose–response relationship and explore the mechanisms underlying the rise in the Ca^{2+} signal. We also present evidence that the efflux of Ca^{2+} via the plasma membrane Ca^{2+} pump might be significantly involved in the decay of the BHQ-induced $[Ca^{2+}]_i$ transient.

2. Methods

2.1. Cell culture

MDCK cells obtained from the American Type Culture Collection (CRL-6253, MD, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2-containing humidified air.

2.2. Solutions

Normal Ca^{2+} medium (pH 7.4) contained (in mM): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 10 and glucose 5. Ca^{2+}-free medium contained no added Ca^{2+} plus 1 mM EGTA. Nominally Ca^{2+}-free medium contained no added Ca^{2+} and no EGTA. The experimental solution contained less than 0.1% of solvent (dimethyl sulfoxide or ethanol), which did not affect $[Ca^{2+}]_i$ ($n = 3$).

2.3. Optical measurements of $[Ca^{2+}]_i$

Trypsinized cells (10^6/ml) were loaded with the ester form of fura-2, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2 -(2′-amino-5′-methylphenoxy) -ethane-*N*,*N*,*N*,*N*-tetraacetic acid pentaacetoxymethyl ester (fura-2/AM; 2 μM), for 30 min at 25°C in DMEM. Cells were washed and resuspended in normal Ca^{2+} medium and were washed every hour during experiments to minimize extracellular dye. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette contained 1 ml of normal medium and 0.5 million of cells unless otherwise stated. Fluorescence was monitored with a Hitachi F-4500 spectrofluorophotometer (Japan) by continuously recording excitation signals at 340 and 380 nm and emission signals at 510 nm at 1-s intervals. Maximum and minimum fluorescences were obtained by adding Triton-100 (0.1%) and EGTA (20 mM) sequentially at the end of the experiment. The ratio of excitation signals at 340 and 380 nm was used to calculate $[Ca^{2+}]_i$ as described previously (Grynkiewicz et al., 1985), assuming a K_d of 155 nM. Preliminary experiments showed that trypsinized cells yielded qualitatively similar results as cells attached to coverslips. We decided to use trypsinized cells because this procedure is easier and less time-consuming.

2.4. Chemical reagents

The reagents for cell culture were from Gibco (Grand Island, NY, USA). Fura-2/AM was from Molecular Probes (Eugene, OR, USA). BHQ and 1-[β-[3-(4-methoxyphenyl)-propoxy]-4-methoxyphenethyl]-1*H*-imidazole hydrochloride (SKF 96365) were from Biomol (Plymouth Meeting, PA, USA). All other reagents were from Sigma (St. Louis, MO, USA).

2.5. Statistical analyses

All values are reported as the means ± S.E. ($n = 3$). Statistical comparisons were determined by using Student's paired *t*-test, and significance was accepted when p was < 0.05.

3. Results

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

In normal Ca^{2+} medium BHQ evoked a transient increase in $[Ca^{2+}]_i$ which showed a slow rise, a gradual decay and a sustained plateau phase (Fig. 1A). The magnitude of the Ca^{2+} signal depended on the concentration of BHQ between 10 μM and 0.2 mM. The Ca^{2+} signal became saturated at 0.1–0.2 mM BHQ. The increase in $[Ca^{2+}]_i$ was slower in response to lower concentrations of BHQ. Bradykinin and ATP have been shown to trigger marked increases in $[Ca^{2+}]_i$ in MDCK cells (Aboolian et

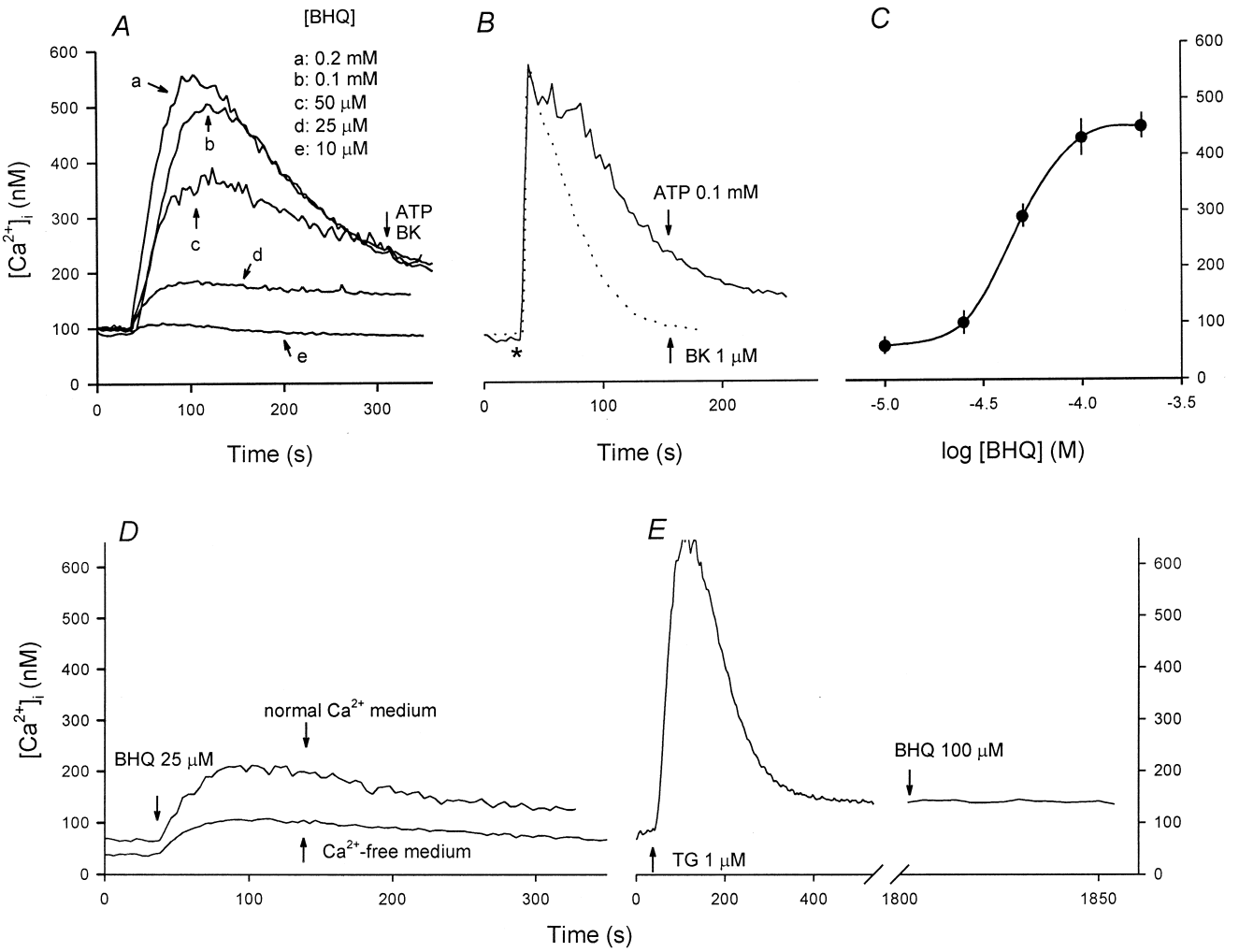

Fig. 1. (A) Dose-dependent effect of BHQ on $[Ca^{2+}]_i$ in normal Ca^{2+} medium. BHQ was added at concentrations of 10 μM–0.2 mM. (B) Effects of bradykinin (BK) and ATP on $[Ca^{2+}]_i$ in normal Ca^{2+} medium. The agonist was added at the time indicated by ∗. (C) Dose–response curve plotted from data in (A). The *y*-axis represents the net peak $[Ca^{2+}]_i$ height in nM (baseline of about 100 nM was subtracted). Data are the means ± S.E. of three experiments. (D) Comparison of BHQ (25 μM)-induced Ca^{2+} responses in normal Ca^{2+} medium (*upper trace*) and Ca^{2+}-free medium (*lower trace*). Ca^{2+}-free medium was without added Ca^{2+} plus 1 mM EGTA. (E) Cells were treated with thapsigargin (TG) for 30 min in normal Ca^{2+} medium before addition of BHQ. The traces are typical of three experiments.

al., 1989; Jan et al., 1998a,c), most likely by releasing Ca^{2+} from the IP_3-sensitive endoplasmic reticulum Ca^{2+} store (Pidikiti et al., 1985; Friedrich et al., 1991). Fig. 1B illustrates two $[Ca^{2+}]_i$ transients evoked by bradykinin and ATP, respectively. However, neither bradykinin (1 μM) nor ATP (0.1 mM) increased $[Ca^{2+}]_i$ after cells had been pretreated with 0.1–0.2 mM BHQ for 6 min (Fig. 1A; traces *a* and *b*), suggesting that BHQ depleted the IP_3-sensitive Ca^{2+} store. The relationship between the dose and the peak Ca^{2+} signal for BHQ yielded an EC_{50} of 40 μM (Fig. 1C), calculated by fitting a Hill equation to the data.

3.2. Extracellular Ca^{2+} influx and intracellular Ca^{2+} release contribute equally to the BHQ-induced $[Ca^{2+}]_i$ transient

In many cells, it is recognized that BHQ elevates $[Ca^{2+}]_i$ by releasing endoplasmic reticulum Ca^{2+} followed by capacitative Ca^{2+} entry (Robinson et al., 1992; Leis et al., 1996; Applegate et al., 1997). However, the relative contribution of these two pathways has not been determined in MDCK cells previously. Fig. 1D shows that removal of extracellular Ca^{2+} (no added Ca^{2+} plus 1 mM EGTA) inhibited the 25 μM BHQ-induced $[Ca^{2+}]_i$ transient by about half the peak value and also the area under the curve, and nearly abolished the sustained plateau. Similar results were obtained with 0.1 mM BHQ (not shown).

Although it is well known that BHQ induces capacitative Ca^{2+} entry in many cells, the possibility that BHQ could directly activate a Ca^{2+} influx pathway which is distinguishable from capacitative Ca^{2+} entry cannot be excluded. We tested this possibility by first depleting the endoplasmic reticulum Ca^{2+} store in normal Ca^{2+} medium followed by addition of BHQ to see if BHQ could still

increase $[Ca^{2+}]_i$. We also depleted the Ca^{2+} store by treating cells with another inhibitor of the endoplasmic reticulum Ca^{2+} pump, thapsigargin (Thastrup et al., 1990). Fig. 1E shows that, in the presence of extracellular Ca^{2+}, thapsigargin (1 μM) evoked a $[Ca^{2+}]_i$ transient very similar in kinetics and magnitude to that elicited by BHQ (0.1–0.2 mM). However, BHQ added 30 min afterwards failed to increase $[Ca^{2+}]_i$.

3.3. The BHQ-induced capacitative Ca^{2+} entry is abolished by La^{3+} or Gd^{3+}, and partly inhibited by SKF 96365

We next directly measured the BHQ-induced capacitative Ca^{2+} entry and examined the effects of several Ca^{2+} entry blockers. Fig. 2 shows that in nominally Ca^{2+}-free medium, addition of 10 mM $CaCl_2$ to cells pretreated with BHQ for 6 min induced a Ca^{2+} signal (between 430 and 580 s) with a peak value of 250 ± 13 nM (trace a; $n = 3$). This Ca^{2+} signal was most likely due to capacitative Ca^{2+} entry as a result of the depletion of the BHQ-sensitive internal Ca^{2+} store because addition of Ca^{2+} alone without BHQ pretreatment only increased the basal $[Ca^{2+}]_i$ slightly from 53 ± 20 to 92 ± 13 nM (trace *d*; $n = 3$; $P < 0.05$). This capacitative Ca^{2+} entry was abolished by the general Ca^{2+} entry blocker La^{3+} (0.1 mM) or Gd^{3+} (50 μM) added 30 s prior to Ca^{2+} (trace *c*). La^{3+} or Gd^{3+} did not significantly alter the basal $[Ca^{2+}]_i$ levels. The area under the curve of the capacitative Ca^{2+} entry response was inhibited by $40 \pm 4\%$ (trace *b*; $n = 3$; $P < 0.05$) by 50 μM SKF 96365, a Ca^{2+} blocker commonly used to inhibit capacitative Ca^{2+} entry in a number of cells including MDCK cells (Merritt et al., 1990; Jan et al., 1998b).

3.4. Mechanism of decay of the BHQ-induced $[Ca^{2+}]_i$ increase

So far, we have elucidated the mechanisms underlying the rising phase of the BHQ-induced $[Ca^{2+}]_i$ increase. We next examined the mechanism underlying the decay of the $[Ca^{2+}]_i$ increase. The decay of an increase in $[Ca^{2+}]_i$ in most cells involves buffering by Ca^{2+}-binding proteins, efflux via plasma membrane Ca^{2+} pumps or Na^+/Ca^{2+} exchange, and buffering by mitochondria (Clapman, 1995). Buffering by the nucleus might also play a significant role (Al-Mohanna et al., 1994; Bkaily et al., 1996). We investigated the roles of Ca^{2+} efflux via plasma membrane Ca^{2+} pumps or Na^+/Ca^{2+} exchange, and buffering by mitochondria. For plasma membrane Ca^{2+} pumps, a selective inhibitor is not available. We tried two manipulations which have been reported to inhibit the Ca^{2+} pump: addition of La^{3+} or extracellular alkalization to pH 8 (Milanick, 1990). Eosin was also shown to inhibit the Ca^{2+} pump (Gatto and Milanick, 1993), but we found that at 1 μM eosin interfered with fura-2 fluorescence measurements and thus it could not be tested in our experiments. Fig. 3A shows that when La^{3+} (0.1 mM) was added 30 s prior to BHQ in normal Ca^{2+} medium, both the peak value and the area under the curve of the BHQ response were increased by $40 \pm 5\%$ ($n = 3$; $P < 0.05$). La^{3+} did not alter the basal $[Ca^{2+}]_i$. We tested whether the effect of La^{3+} was mimicked by another lanthanide, Gd^{3+}. Fig. 3B shows that, similar to La^{3+}, Gd^{3+} (50 μM) added 30 s prior to BHQ increased the area under the curve of the BHQ response by $75 \pm 7\%$ ($n = 3$; $P < 0.05$). We next examined the effect of extracellular alkalization on the BHQ-induced Ca^{2+} response. Fig. 3C shows that alkalization (pH 8) increased the BHQ response by $37 \pm 7\%$ in

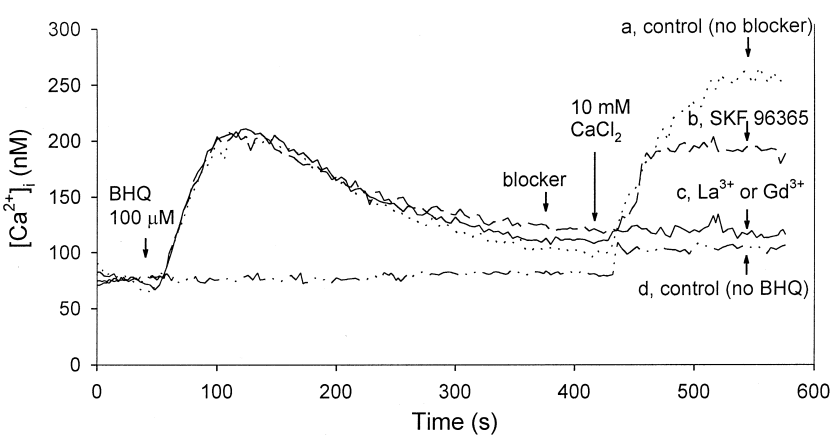

Fig. 2. Effects of several Ca^{2+} blockers on BHQ-induced capacitative Ca^{2+} entry. The experiments were carried out in nominally Ca^{2+}-free medium. BHQ was added for 7 min to deplete the endoplasmic reticulum Ca^{2+} store. $CaCl_2$ (10 mM) was added afterwards to induce capacitative Ca^{2+} entry. Trace *a*, control with BHQ pretreatment without addition of blocker prior to Ca^{2+}. Trace *d*, control with addition of Ca^{2+} without BHQ pretreatment. In traces *b* and *c*, a blocker was added 30 s prior to addition of Ca^{2+}. The blocker in trace *b* was 50 μM SKF 96365, and in trace *c* was 100 μM $LaCl_3$ or 50 μM $GdCl_3$. The traces are typical of three experiments.

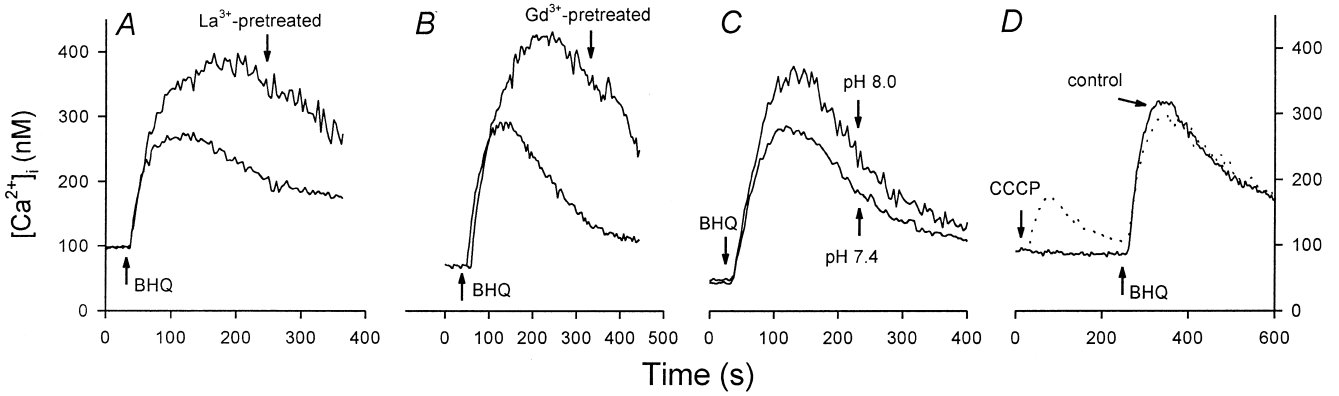

Fig. 3. (A) *Upper trace*: 100 μM La^{3+} was added 30 s prior to addition of BHQ. *Lower trace*: control without addition of La^{3+}. (B) *Upper trace*: Gd^{3+} was added 30 s prior to addition of BHQ. *Lower trace*: control without addition of Gd^{3+}. (C) *Upper trace*: BHQ was added to cells suspended in Ca^{2+} medium of pH 8. *Lower trace*: control with cells suspended in normal Ca^{2+} medium (pH 7.4). (D) *Dotted trace*: 2 μM CCCP was added at 30 s followed by BHQ added at 260 s. *Solid trace*: control without CCCP pretreatment. All the experiments were performed in the presence of extracellular Ca^{2+}. The concentration of BHQ in A–D was 50 μM. The traces are typical of three experiments.

peak value and by 26 ± 6% in the area under the curve ($n = 3$; $P < 0.05$).

We also examined the role of mitochondria in the decay of the BHQ response. Fig. 3D shows that 2 μM of the mitochondrial uncoupler carbonylcyanide *m*-chlorophenylhydrazone (CCCP) induced a small $[Ca^{2+}]_i$ transient with a peak value less than 200 nM (*dash line*), possibly reflecting the release of Ca^{2+} stored in resting mitochondria. We have reported similar effects of CCCP previously (Jan et al., 1998b,c). Subsequently added BHQ elicited a Ca^{2+} signal not significantly different from the control signal (*solid line*; without CCCP pretreatment) ($n = 3$; $P > 0.05$). If the decay of the BHQ response was due to buffering by mitochondria, then when mitochondria were uncoupled by CCCP, the BHQ response should have been more pronounced than the control response. Similarly, pretreatment with another mitochondrial inhibitor, oligomycin (5 μg/ml), for 3 min did not alter the BHQ-induced Ca^{2+} signal (not shown).

Active Na^+/Ca^{2+} exchange has been reported in MDCK cells (Snowdowne and Borle, 1985; Jan et al., 1998b,c). We have recently shown that Na^+/Ca^{2+} exchange does not play a role in the decay of the Ca^{2+} signal evoked by bradykinin (Jan et al., 1998c) or U73122 (Jan et al., 1998b). To examine the role of Na^+/Ca^{2+} exchange in the decay of the BHQ-induced $[Ca^{2+}]_i$ increase, we lowered the Na^+ concentration of the extracellular medium by adding 5 μl of concentrated cell suspension to 2 ml of Na^+-free medium in a cuvette immediately before $[Ca^{2+}]_i$ was measured. Na^+ was replaced by *N*-methyl-glucamine but there was no change in osmolarity. This yielded an extracellular Na^+ concentration of 0.38 mM. The density of the cells was maintained at 0.5 million/ml as in the other experiments. As we have shown previously, lowering extracellular Na^+ to 0.38 mM evoked a gradually rising $[Ca^{2+}]_i$ transient which reached a peak of about 1 μM $[Ca^{2+}]_i$, and then slowly decayed to near prestimulatory levels in 15 min (Jan et al., 1998b,c). BHQ added subsequently induced a $[Ca^{2+}]_i$ transient that was not much different from the control transient in magnitude and kinetics (not shown).

4. Discussion

In this study we have investigated the mechanism of the rise and decay of the BHQ-induced $[Ca^{2+}]_i$ transient in MDCK cells. Fig. 1D shows that extracellular Ca^{2+} influx and intracellular Ca^{2+} release contributed equally to the BHQ-induced $[Ca^{2+}]_i$ increase in Ca^{2+} medium, and that the plateau phase of the BHQ response was mainly maintained by the influx of extracellular Ca^{2+}.

Our data in Fig. 1E suggest that BHQ and thapsigargin share similar internal Ca^{2+} stores and that once the IP_3-sensitive Ca^{2+} store has been depleted, BHQ cannot directly induce Ca^{2+} influx through the capacitative Ca^{2+} entry or another Ca^{2+} influx pathway. Thus the BHQ-induced Ca^{2+} influx that we observed (Fig. 1D) was most likely due to capacitative Ca^{2+} entry without involvement of a direct Ca^{2+} influx pathway. Collectively, the source of Ca^{2+} for the BHQ-induced $[Ca^{2+}]_i$ increase is initially the endoplasmic reticulum, followed by capacitative Ca^{2+} entry as a result of the depletion of the endoplasmic reticulum Ca^{2+} store.

The reason that the measurements of capacitative Ca^{2+} entry were performed in nominally Ca^{2+}-free medium instead of EGTA-containing Ca^{2+}-free medium was because EGTA could chelate La^{3+}, as it has a higher affinity for La^{3+} than for Ca^{2+} (Martell and Smith, 1976). Indeed, no inhibition by La^{3+} or Gd^{3+} of the BHQ-induced capacitative Ca^{2+} entry was found when the experiments were performed in Ca^{2+}-free medium containing 1 mM EGTA (not shown). Similar to BHQ, thapsigargin also induced capacitative Ca^{2+} entry which was abolished by

La^{3+} or Gd^{3+} and was partly inhibited by SKF 96365 (not shown). Using similar experimental protocols, we have also found that La^{3+} abolishes ATP- or U73122-induced capacitative Ca^{2+} entry (Jan et al., 1998a,b) and that SKF 96365 partly inhibits the U73122-induced capacitative Ca^{2+} entry (Jan et al., 1998b). It has also been shown that La^{3+} and SKF 96365 inhibit the thapsigargin-induced capacitative Ca^{2+} currents in MDCK cells (Dietl et al., 1996).

We also examined the mechanism underlying the recovery of the BHQ-induced $[Ca^{2+}]_i$ transient. Our data suggest that the plasma membrane Ca^{2+} pump might play a significant role in lowering the BHQ-induced $[Ca^{2+}]_i$ increase, based on the observations that inhibition of the Ca^{2+} pump with lanthanides or alkalization of the extracellular medium enhanced the BHQ response. Similarly, we have found that efflux via the plasma membrane Ca^{2+} pump contributes significantly to the thapsigargin- or bradykinin-induced Ca^{2+} signal in MDCK cells (Jan et al., 1998c). Because La^{3+} completely diminished the BHQ-induced Ca^{2+} influx (Fig. 2), the BHQ-induced $[Ca^{2+}]_i$ increase observed in the presence of La^{3+} (Fig. 3A) was likely due to the release of Ca^{2+} from the endoplasmic reticulum Ca^{2+} store. Therefore, these data indicate that the Ca^{2+} released from the endoplasmic reticulum upon BHQ stimulation mainly left the cells via the plasma membrane Ca^{2+} pump. As we discussed previously (Jan et al., 1998a,c), it is unlikely that this action of La^{3+} was an artifact because La^{3+} did not elevate the basal $[Ca^{2+}]_i$ nor did it enter the cell and alter the IP_3-dependent Ca^{2+} signaling pathway. The most plausible explanation for the action of La^{3+} is, in addition to blockade of capacitative Ca^{2+} entry, inhibition of the plasma membrane Ca^{2+} pump, leading to a delayed efflux of the Ca^{2+} released from the intracellular store. No information is revealed from our results about whether the Ca^{2+} that entered the cell via capacitative Ca^{2+} entry also left the cell via the plasma membrane Ca^{2+} pump. Notably, Gd^{3+}, a lanthanide which also abolished the BHQ-induced capacitative Ca^{2+} entry, mimicked the action of La^{3+} on the BHQ-induced $[Ca^{2+}]_i$ increase. Because Gd^{3+} can block a variety of cation channels, such as Ca^{2+} channels, Na^+ channels, and stretch-activated cation channels (Bleakman et al., 1995; Cunningham et al., 1995; Liu et al., 1989), it is possible that, similar to La^{3+}, Gd^{3+} pretreatment potentiated the BHQ-induced $[Ca^{2+}]_i$ transient via inhibition of the plasma membrane Ca^{2+} pump, leading to a delayed decay of the BHQ-induced $[Ca^{2+}]_i$ increase.

The decay of the BHQ-induced $[Ca^{2+}]_i$ increase hardly involved buffering by mitochondria, because pretreatment with CCCP or oligomycin did not alter the BHQ response. We have also found that the bradykinin-induced Ca^{2+} signal in MDCK cells is not affected by CCCP pretreatment (Jan et al., 1998c). Conversely, mitochondrial sequestration plays an important role in the U73122-induced Ca^{2+} signal (Jan et al., 1998b). Since the U73122-induced Ca^{2+} signal was much larger in magnitude than that induced by BHQ or bradykinin, it is possible that mitochondria do not actively sequester cytosolic Ca^{2+} until $[Ca^{2+}]_i$ reaches a certain threshold.

The contribution of Na^+/Ca^{2+} exchange in the decay of the BHQ response is negligible. Similarly, the bradykinin- or U73122-evoked Ca^{2+} signal was not altered by lowering the extracellular Na^+ concentration to 0.38 mM (Jan et al., 1998b,c).

In sum, in the present study we carefully characterized the BHQ-induced Ca^{2+} signal in MDCK cells and examined the mechanism underlying its rise and decay. We found that BHQ increased $[Ca^{2+}]_i$ in a dose-dependent manner, by releasing Ca^{2+} from the endoplasmic reticulum Ca^{2+} store followed by capacitative Ca^{2+} entry. BHQ did not directly trigger Ca^{2+} influx from the extracellular medium. The BHQ-induced capacitative Ca^{2+} entry was abolished by La^{3+} or Gd^{3+} and inhibited by SKF 96365. The decay of the BHQ-induced Ca^{2+} signal might be governed by the efflux of Ca^{2+} via the plasma membrane Ca^{2+} pump.

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