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Agonist-sensitive and -insensitive intracellular Ca^{2+} pools

Separate Ca^{2+}-releasing mechanisms revealed by manoalide and benzohydroquinone

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The mechanism of action of a novel compound, 2,5-di-(t-butyl)-1,4-benzohydroquinone (BHQ), used to modulate cell free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was studied in AR42J cells and pancreatic acini by using single-cell fluorescence techniques applied to Fura-2-loaded cells. In the presence of extracellular Ca^{2+} (Ca^{2+}_{out}), BHQ induced a biphasic $[Ca^{2+}]_i$ increase, an initial and rapid transient followed by a sustained increase. The initial increase was due to Ca^{2+} release from intracellular stores, being independent of Ca^{2+}_{out}. The sustained response was due to Ca^{2+} entry, being dependent on Ca^{2+}_{out}, blocked by La^{3+} and correlated with an increased rate of Mn^{2+} entry, all indicative of increased plasma-membrane permeability to Ca^{2+}. Treatment of AR42J cells with BHQ for about 5 min reversibly blocked agonist-dependent Ca^{2+} release and oscillations, whereas agonist pretreatment decreased, but did not prevent, the effects of BHQ on $[Ca^{2+}]_i$. Accordingly, depletion of the Ins(1,4,5)P_3-mobilizable pool in permeabilized AR42J cells by BHQ required 5 min of incubation, although inhibition of the internal Ca^{2+} pump by BHQ was rapid. These observations suggest that BHQ mobilized an additional intracellular Ca^{2+} pool that did not respond to changes in Ins(1,4,5)P_3. Manoalide, an inhibitor of Ca^{2+} channels, inhibited agonist-evoked $[Ca^{2+}]_i$ oscillation and $[Ca^{2+}]_i$ increase in a dose- and time-dependent manner without significant effect on internal Ca^{2+} pumps and Ca^{2+} content of the internal stores. Manoalide also inhibited the BHQ-evoked $[Ca^{2+}]_i$ increase in the absence and presence of Ca^{2+}_{out}. Neither BHQ nor manoalide affected Ins(1,4,5)P_3 levels in resting or stimulated cells. Therefore, the effect of BHQ appears to involve unmasking of passive Ca^{2+}-permeation pathways in the plasma and intracellular membranes that do not respond to cholecystokinin octapeptide, following its described inhibition of the internal-store Ca^{2+} pumps responsible for accumulating Ca^{2+} in these pools.

INTRODUCTION

Mobilization of Ca^{2+} across the plasma and intracellular membranes is a major pathway of stimulus–response coupling in many cell types [1–4]. Ca^{2+} release from intracellular stores is responsible for the initial rapid increase in $[Ca^{2+}]_i$, which is thought to be mediated by the generation of Ins(1,4,5)P_3 in the cytosol [5]. Mobilization of extracellular Ca^{2+} due to activation of receptor-operated Ca^{2+} entry pathways is responsible for steady-state elevation of $[Ca^{2+}]_i$ [3,4]. This pathway is also responsible for reloading of the intracellular pools [4] and maintaining $[Ca^{2+}]_i$ oscillation [5,6]. These pathways, which increase $[Ca^{2+}]_i$ in response to cell activation, are counteracted by primary active Ca^{2+} pumps located in both intracellular and plasma membranes. Monoclonal antibodies [7] and cloning techniques [8] have identified several isoforms of the intracellular Ca^{2+} pumps which are thought to be located in separate Ca^{2+} stores [6,7]. Activation of these various pathways, which differ in relative importance in different cell types and in terms of responses to different agonists [4,9,10], results in a characteristic biphasic change in $[Ca^{2+}]_i$ observed in non-excitable cells [4] and can also lead to oscillatory rather than steady-state changes in $[Ca^{2+}]_i$, [5,6].

Studies designed to elucidate the relative importance of the various Ca^{2+}-regulatory systems have been hampered by the lack of specific inhibitors of Ca^{2+} pumps or passive Ca^{2+} pathways. However, a tool which has become popular recently in studying Ca^{2+} homoeostasis in cells is the inhibition of the intracellular Ca^{2+} pump by compounds such as the sesquiterpene lactone thapsigargin [11] and the benzohydroquinone BHQ [12]. Both of these compounds were found to be potent inhibitors of intracellular Ca^{2+} pumps [13,14] without affecting the Ca^{2+} pumps located in the plasma membrane or passive Ca^{2+} pathways [13,14]. Hence changes in $[Ca^{2+}]_i$ brought about by the addition of these agents have been ascribed to inhibition of the Ca^{2+}-scavenging action of the intracellular pumps [11,12,15,16]. From studying the effects of these compounds on agonist-evoked $[Ca^{2+}]_i$ increase, it was further suggested that these compounds mobilize the agonist-sensitive pool to increase $[Ca^{2+}]_i$ [12,15–17].

In the present work, we have used the pancreatic tumour cell line AR42J and freshly isolated pancreatic acini to determine the sites of interaction of BHQ with Ca^{2+}-regulatory mechanisms. We provide evidence that treatment with BHQ results in mobilization of Ca^{2+} from two separate intracellular pools, an agonist-sensitive and an agonist-insensitive pool. Ca^{2+} mobilization from the agonist-insensitive pool is rapid and accounts for the initial BHQ-evoked $[Ca^{2+}]_i$ increase, whereas Ca^{2+} mobilization from the agonist-sensitive pool is slow and results in time-dependent inhibition of agonist-evoked $[Ca^{2+}]_i$ increase. BHQ appears to increase $[Ca^{2+}]_i$ rapidly by unmasking the activity of a Ca^{2+} channel after the inhibition of internal Ca^{2+} pumps.

METHODS

Culture conditions

AR42J cells were plated on 75 cm^2 plastic tissue-culture plates at a density of 25000/cm^2 and cultured at 37 °C in a humidified

Abbreviations used: $[Ca^{2+}]_i$, free cytosolic Ca^{2+} concentration (similarly $[Mn^{2+}]_i$); Ca^{2+}_{out}, extracellular Ca^{2+}; BHQ, 2,5-di-(t-butyl)-1,4-benzohydroquinone; CCK-OP, cholecystokinin octapeptide; CCK-JMV, Box-Tyr(SO_3)-Nle-Gly-Trp-Nle-Asp-2-phenethyl ester; TCA, trichloroacetic acid.

air/CO_2 (19:1) atmosphere in Ham's F12/Dulbecco's modified Eagle's medium (1:1, v/v) supplemented with 14.3 mM-$NaHCO_3$, 1.2 mM-L-glutamine, 7.5% (v/v) fetal-calf serum, 0.1 mg of streptomycin/ml and 100 units of penicillin/ml. The cells were fed twice per week and reached maximal growth within 10–12 days in culture. For $[Ca^{2+}]_i$ measurements, cells released from culture plates were seeded at the middle of thin glass cover-slips placed in Petri dishes at the same density as above. After 3 h incubation, the cover slips were washed to remove unattached cells and then covered with tissue-culture medium. The cells were used after 2–5 days in culture. On the day of the experiment, the cells were washed twice with serum-free tissue-culture medium and then incubated in the same medium containing 2 μM-Fura 2/AM for 45–60 min at 37 °C. Cells were washed once and maintained in serum-free medium at room temperature until use.

Preparation of acini

Dispersed pancreatic acini were prepared from rat pancreas by two cycles of collagenase digestion as described previously [18,19]. The acini from one pancreas were suspended in 10 ml of serum-free tissue-culture medium containing 1 mg of BSA/ml and 0.01% (w/v) soybean trypsin inhibitor (incubation solution) and kept on ice until use. A 2 ml portion of acini suspension was used for each loading with Fura 2. The acini were placed in incubation solution containing 5 μM-Fura 2/AM for 30 min at 37 °C. After completion of Fura 2 loading, the acini were washed once and resuspended in a final volume of 10 ml of incubation solution. The Fura 2-loaded acini were placed on ice until attachment to cover-slips. The acini were then plated on thin glass cover-slips coated with a thin film of polylysine and allowed to attach for 20–30 min at room temperature before the cover-slip was mounted in the perfusion chamber. The acini were perfused with solution A [140 mM-NaCl, 5 mM-KCl, 1 mM-$MgCl_2$, 10 mM-Hepes (pH 7.4 with NaOH), 10 mM-glucose and 0.1% BSA) at 37 °C for at least 10 min before a challenge with agonist or BHQ.

Fluorescence recording from single cells

The cover-slips with AR42J cells or pancreatic acini attached to them were mounted in an open perfusion chamber. The perfusate volume in the chamber was adjusted to 0.3 ml. The perfusate, placed in 60 ml syringes, was delivered through an eight-way valve to spiral-shaped glass tubing that was water-jacketed and kept at 37 °C. From the glass tubing, a warm perfusate was delivered to the chamber, which was also heated by means of a circulating water bath that allowed water flow through metal tubing which fitted in the bottom of the chamber. The cells were continuously perfused at a flow rate of 10–12 ml/min with solution A, supplemented with 1 mM-$CaCl_2$ unless otherwise specified. The recording system included a Nikon Diaphot inverted microscope equipped with a CL Fluor 40× oil-immersion objective attached to a Photon Technology International Delta Scan 1 spectrofluorimeter which provided dual-wavelength excitation light. The excitation light (340 or 360 and 380 nm wavelengths) was selected by a spinning chopper mirror and directed to the sample by a dichroic mirror. The emitted light of 510 nm wavelength was monitored at a resolution of 3 s^{-1} by a photomultiplier tube. The light monitored at an excitation wavelength of 340 and 380 nm was used to calculate the 340/380 ratio, and the ratio was converted into $[Ca^{2+}]_i$ as described previously [20].

Determination of $[Ca^{2+}]_i$ in cell suspension

AR42J cells were released from tissue-culture flasks by treatment with trypsin (0.05%) and EDTA (0.02%) in Dulbecco's phosphate-buffered saline. Trypsin/EDTA treatment took place at 37 °C, followed by a rapid 25-fold dilution with solution A and washing twice by centrifugation at 600 *g* for 3 min at 25 °C. Cells were suspended in solution A and incubated with 2.5 μM-Fura 2/AM in a shaking water bath at 37 °C for 30 min. After completion of Fura 2 loading, the cells were washed in solution A and resuspended in this medium. Approx. 10^5 cells/ml in a volume of 50 μl were transferred to 1.95 ml of warm BSA-free solution A, and fluorescence measurements were made while the cells were continuously stirred and maintained at 37 °C. Fluorescence was measured with a Photon Technology International-Delta Scan 1 fluorimeter at excitation wavelengths of 340 and 380 nm and emission wavelength of 500 nm. The 340/380 ratio was used to calculate $[Ca^{2+}]_i$ as indicated above.

Ca^{2+}-flux measurements

AR42J cells were released from tissue-culture flasks by trypsin/EDTA treatment as described above and washed twice with solution B, containing (in mM) KCl 140, $MgCl_2$ 3, Hepes (pH 7.2 with NaOH at 37 °C), $CaCl_2$ 0.01, and sufficient EGTA to adjust $[Ca^{2+}]$ to 0.15 μM. In all solutions used for ^{45}Ca fluxes, Ca^{2+} was measured with the tetra-K^+ form of Fura 2 as detailed previously [21]. The cells were incubated in solution B containing 0.0075% saponin for 5 min at 37 °C and then washed once with solution B. Ca^{2+} uptake was initiated by addition of permeabilized cells to uptake medium (approx. 3.5×10^5 cells/ml), composed of solution B and also containing: antimycin A, 10 μM; oligomycin, 5 μM; $^{45}Ca^{2+}$ (approx. 5×10^5 c.p.m./ml); ATP, 2 mM; with or without 25 μM-BHQ as specified. After 5 min incubation at 37 °C, samples of cells incubated without BHQ were transferred to tubes containing BHQ, Ins(1,4,5)P_3 or both to yield the desired final concentrations. At the indicated times, Ca^{2+} fluxes were terminated by transferring 100 μl portions of cells to tubes containing 2 ml of cold solution B containing 1 mM-$LaCl_3$. The cells were then transferred to filters and washed with 3 × 2.5 ml of cold solution B containing 1 mM-$LaCl_3$. The filters were dissolved in scintillation fluid and counted for $^{45}Ca^{2+}$. When the effect of manoalide on Ca^{2+} uptake was tested, cells released from culture plates and resuspended in BSA-free solution A were incubated with 1.5 μM-manoalide for 5 min at 37 °C. Then the cells were permeabilized and Ca^{2+} uptake was measured as described above, except that all solutions contained 1.5 μM-manoalide.

Mass measurement of Ins(1,4,5)P_3

Ins(1,4,5)P_3 concentration in TCA extracts was measured by displacement of bound [^{3}H]Ins(1,4,5)P_3 essentially as previously described [22,23], except that brain microsomes were used as the source of Ins(1,4,5)P_3-binding protein. AR42J cells grown on six-well culture plates were treated with test compounds as specified. Incubation was terminated by aspiration of incubation medium and addition of 0.5 ml of 10% (w/v) TCA. After incubation for 5–10 min, the TCA extract was removed to clean tubes, supplemented with 0.5 mM-ATP and washed five times with water-saturated diethyl ether. The pH of the washed extracts was adjusted to 8.2 with Tris. In parallel, samples of Ins(1,4,5)P_3 were added to 0.5 ml of 10% TCA containing 0.5 mM-ATP and washed as described above. These samples were used to construct the standard [^{3}H]Ins(1,4,5)P_3-displacement curves. A 300 μl portion of sample or TCA-extracted buffer containing standard amounts of Ins(1,4,5)P_3 was supplemented with approx. 20000 c.p.m. of [^{3}H]Ins(1,4,5)P_3 and added to 300 μl of brain microsomes. The mixtures were incubated in an ice-cold water bath for 10 min, after which the samples were centrifuged for 10 min at 12000 *g* in a cold Eppendorf centrifuge. The pellets were used to measure bound [^{3}H]Ins(1,4,5)P_3. Ins(1,4,5)P_3 content of each sample was determined from the standard curve.

Analysis of results

All experimental protocols presented were repeated at least three times with cells from different sub-passages or different preparations of acini. Where appropriate, results are reported as means ± S.E.M.

RESULTS

BHQ effects on passive Ca^{2+} pathways

Fig. 1 illustrates the effect of BHQ on $[Ca^{2+}]_i$ recorded from single AR42J cells loaded with Fura 2. Preliminary experiments showed that the effect of BHQ on $[Ca^{2+}]_i$ was dose-dependent and maximized at 20 μM. Therefore 25 μM-BHQ was used in all experiments to ensure maximal effect. Exposing AR42J cells to BHQ resulted in a transient $[Ca^{2+}]_i$ increase in the presence (Fig. 1*a*) and absence (Fig. 1*b*) of Ca^{2+}_{out}. In the presence of Ca^{2+}_{out}, $[Ca^{2+}]_i$ increased from a resting value of 96 ± 8 nM ($n = 42$) to 875 ± 31 nM ($n = 16$) within 5 s and then stabilized at 289 ± 38 nM ($n = 12$). Removal of Ca^{2+}_{out} decreased $[Ca^{2+}]_i$ back to near-resting levels (Fig. 1*a*). In the absence of Ca^{2+}_{out}, there was still a $[Ca^{2+}]_i$ transient, with $[Ca^{2+}]_i$ rising to 615 ± 47 nM ($n = 4$) followed by a fall to basal levels within 2 min of incubation. Restoring Ca^{2+} to the incubation medium resulted in a rapid $[Ca^{2+}]_i$ increase and stabilization of $[Ca^{2+}]_i$ at 310 ± 43 nM.

It was previously suggested that in hepatocytes the sustained $[Ca^{2+}]_i$ increase caused by BHQ does not reflect activation of Ca^{2+} entry, but rather a slow Ca^{2+} efflux across the plasma membrane [12]. Therefore it was necessary to verify by independent means that the effect of Ca^{2+}_{out} on the BHQ response shown in Fig. 1 represents stimulation of Ca^{2+} entry. This is shown in Fig. 2, in which we tested the effect of La^{3+} on the BHQ-evoked $[Ca^{2+}]_i$ increase and the ability of BHQ to stimulate Mn^{2+} entry into AR42J cells. After a control to demonstrate the effect of BHQ on $[Ca^{2+}]_i$ in the absence and then in the presence of Ca^{2+}_{out}, the cell was perfused with Ca^{2+}-free medium. Including 50 μM-La^{3+} in the medium ablated the $[Ca^{2+}]_i$ increase observed upon re-admission of Ca^{2+}_{out}. This effect was reversible, since removal of La^{3+} resulted in the $[Ca^{2+}]_i$ increase expected from resumption of Ca^{2+} entry (Fig. 2*a*). Fig. 2(*b*) shows the effect of BHQ on Mn^{2+} entry. The signal recorded at an excitation wavelength of 380 nm reports changes in $[Ca^{2+}]_i$ and $[Mn^{2+}]_i$, whereas that recorded at 360 nm excitation reports exclusively Mn^{2+} entry. Exposure of the cells to 25 μM-BHQ augmented the rate of Mn^{2+} entry when compared with the rate measured during the control period before BHQ addition.

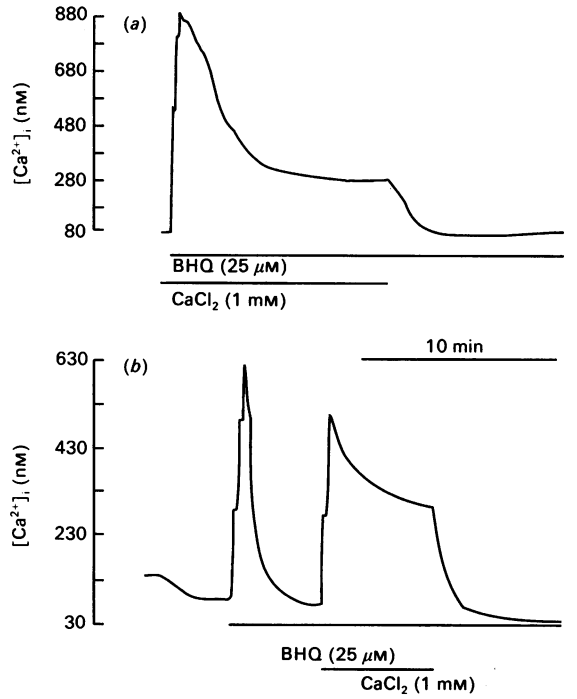

Fig. 1. Effect of BHQ on $[Ca^{2+}]_i$ in the presence and absence of Ca^{2+}_{out}

AR42J cells loaded with Fura 2 were perfused with solution A containing 1 mM-$CaCl_2$. The cell shown in panel (*a*) was then exposed to the same medium containing 25 μM-BHQ. Where indicated, the cell was perfused with solution B (Ca^{2+}-free solution A containing 0.1 mM-EGTA) and 25 μM-BHQ. The cell shown in panel (*b*) was perfused with solution B before perfusion with solution B containing 25 μM-BHQ. Then the cell was exposed to Ca^{2+}_{out} by perfusion with solution A containing 25 μM-BHQ.

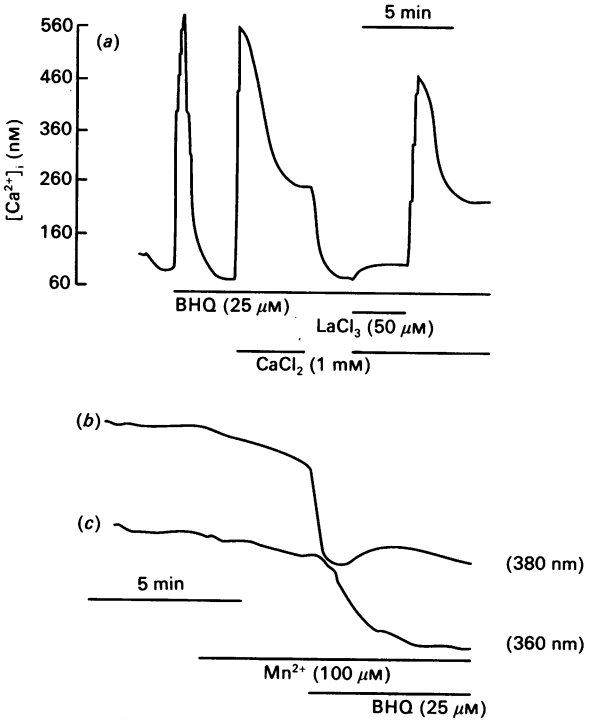

Fig. 2. Activation of Ca^{2+} entry by BHQ in AR42J cells

In experiment (*a*) the cell was perfused with solutions of the indicated compositions. Ca^{2+}-containing solution was solution A and Ca^{2+}-free solution was solution B; 50 μM-La^{3+} was included in solution A. When La^{3+} was removed, solution A contained 1.1 mM-$CaCl_2$ and 0.1 mM-EGTA to facilitate the removal of La^{3+} from the perfusion chamber. In the experiment giving traces (*b*) and (*c*), Fura 2 fluorescence was recorded at excitation wavelengths of 380 nm (*b*) and 360 nm (*c*) from the same cell. The cell was perfused with solution A and, where indicated, with solution A containing 100 μM-Mn^{2+} and then solution A containing Mn^{2+} and 25 μM-BHQ.

An activity in AR42J cells not present in hepatocytes is a plasma-membrane-located voltage-sensitive Ca^{2+} channel [24]. To exclude the possibility that BHQ treatment activated Ca^{2+} entry through modulation of this channel, we tested the effect of the blocker nisoldipine. Complete inhibition of the voltage-sensitive Ca^{2+} channel with nisoldipine did not prevent BHQ-induced Ca^{2+} release from intracellular stores or activation of Ca^{2+} entry (results not shown). To provide further evidence, we tested the effect of BHQ on Ca^{2+} and Mn^{2+} entry in freshly isolated pancreatic acinar cells, which do not express voltage-sensitive Ca^{2+} channels. Fig 3 shows that the effects of BHQ on $[Ca^{2+}]_i$ and $[Mn^{2+}]_i$ in pancreatic acini were essentially identical with those observed in AR42J cells. Thus in the presence of

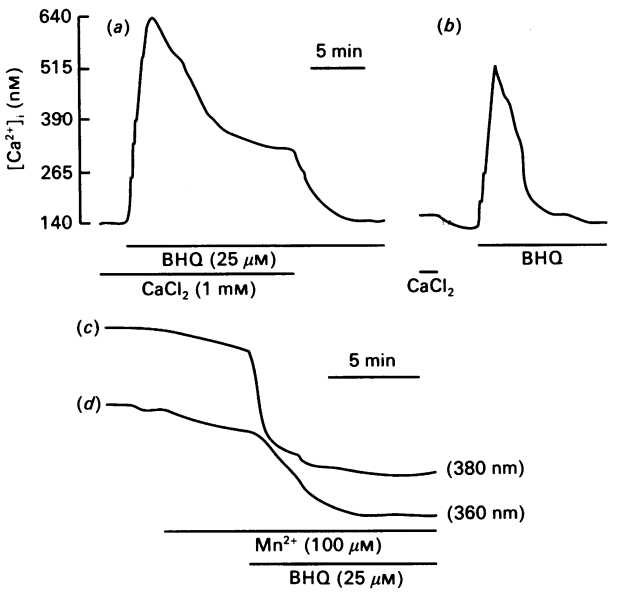

Fig. 3. BHQ-evoked Ca^{2+} release and Ca^{2+} entry in pancreatic acini

Pancreatic acinar cells loaded with Fura 2 were perfused with the indicated solutions. The cell in panel (*a*) was exposed to solution A containing 25 μM-BHQ and then to Ca^{2+}-free solution A containing BHQ. The cell in panel (*b*) was perfused with Ca^{2+}-free solution A before exposure to BHQ. Panels (*c*) and (*d*) record the signal obtained at excitation wavelengths of 380 and 360 nm respectively from a cell perfused with solution A. Where indicated, 100 μM-Mn^{2+} and 25 μM-BHQ were included in the perfusate.

extracellular Ca^{2+}, BHQ induced a biphasic effect on Ca^{2+}, a transient increase (peak 675±84 nM) ($n = 4$) followed by a plateau (290±53 nM) (Fig. 3*a*). In the absence of extracellular Ca^{2+}, BHQ increased $[Ca^{2+}]_i$ to 480±62 nM ($n = 4$), after which $[Ca^{2+}]_i$ was decreased to near resting levels (Fig. 3*b*). Exposure of pancreatic acini to BHQ also augmented the rate of Mn^{2+} entry by approx. 4.6±0.7 ($n = 3$)-fold (Figs 3*c* and 3*d*). Hence it appears that treatment of AR42J cells and pancreatic acini with BHQ results in Ca^{2+} release from intracellular stores, which is followed by augmentation of Ca^{2+} entry.

BHQ-sensitive Ca^{2+} pools

In this set of experiments, the location of the releasable Ca^{2+} was investigated. Fig. 4(*a*) shows that stimulation of AR42J cells with CCK-OP induced a typical transient increase in $[Ca^{2+}]_i$, with a maximum of 970±135 nM ($n = 5$). That this concentration of CCK-OP released all the agonist-mobilizable Ca^{2+} pools was verified by including the maximal concentration of carbachol in the perfusion medium. In a CCK-OP-stimulated cell, carbachol had no further effect on $[Ca^{2+}]_i$. In contrast, when BHQ was added there was a further $[Ca^{2+}]_i$ increase to 612±83 nM ($n = 5$), which was significantly lower than when BHQ was added before any agonist, but similar to that measured with BHQ in the absence of Ca^{2+}_{out} (cf. Fig. 1). Simultaneous exposure of the cells to BHQ and CCK-OP increased $[Ca^{2+}]_i$ to 1620±215 nM ($n = 4$), which was higher than the $[Ca^{2+}]_i$ increase evoked by CCK-OP or BHQ alone (Fig. 4*b*). In this case, the cells decreased $[Ca^{2+}]_i$ back to the basal level in the continuous presence of CCK-OP and BHQ. Fig. 4(*c*) shows the effect of CCK-OP on $[Ca^{2+}]_i$ in a cell treated with BHQ for 9.5 min. In such cells, CCK-OP did not increase $[Ca^{2+}]_i$, but rather decreased it back to resting levels. This could perhaps be accounted for by an activation of the plasma-membrane Ca^{2+} pump by agonists [25,26]. Hence there is a rapidly mobilized pool of intracellular Ca^{2+} sensitive to BHQ but insensitive to Ins(1,4,5)P_3-mediated agonists.

To characterize further the relationship between BHQ and agonist mobilization of Ca^{2+}, we determined the time course of inhibition of the agonist response by BHQ (Fig. 5). Stimulation with CCK-OP of a cell incubated with BHQ for 1 min increased $[Ca^{2+}]_i$ to 775 nM (Fig. 5*a*). Increasing the incubation time with BHQ to 2, 3 and 4.6 min (Figs. 5*b*–5*d*) resulted in an agonist-evoked $[Ca^{2+}]_i$ increase from approx. 235 to 540, 385 and 305 nM

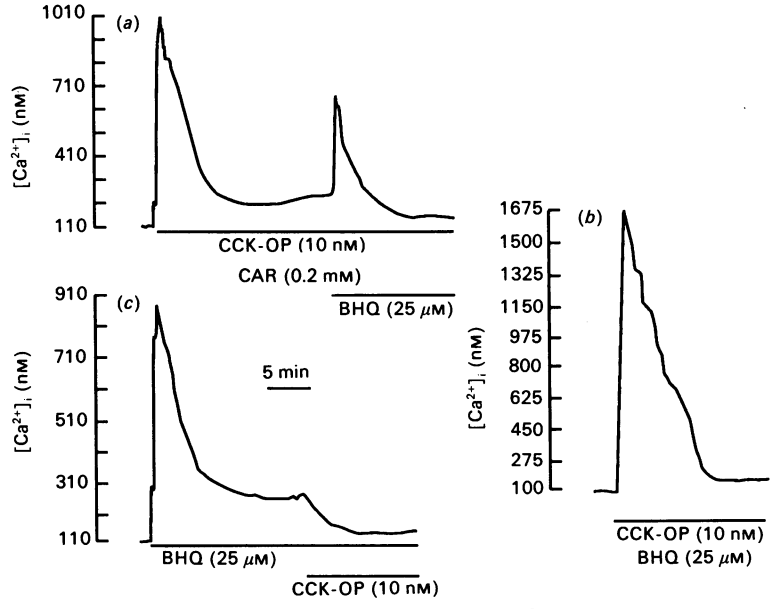

Fig. 4. Effect of simultaneous and sequential stimulation with agonists and BHQ on $[Ca^{2+}]_i$

The cell in (*a*) was stimulated with 10 nM-CCK-OP and, where indicated, 0.2 mM-carbachol (CAR) was included in the perfusion medium to demonstrate the complete depletion of the agonist-mobilizable pool. Where indicated, the cell was exposed to 25 μM-BHQ in the continuous presence of CCK-OP. The cell in (*b*) was simultaneously exposed to CCK-OP and BHQ. In (*c*) the cell was exposed to 25 μM-BHQ for 9.5 min before stimulation with CCK-OP.

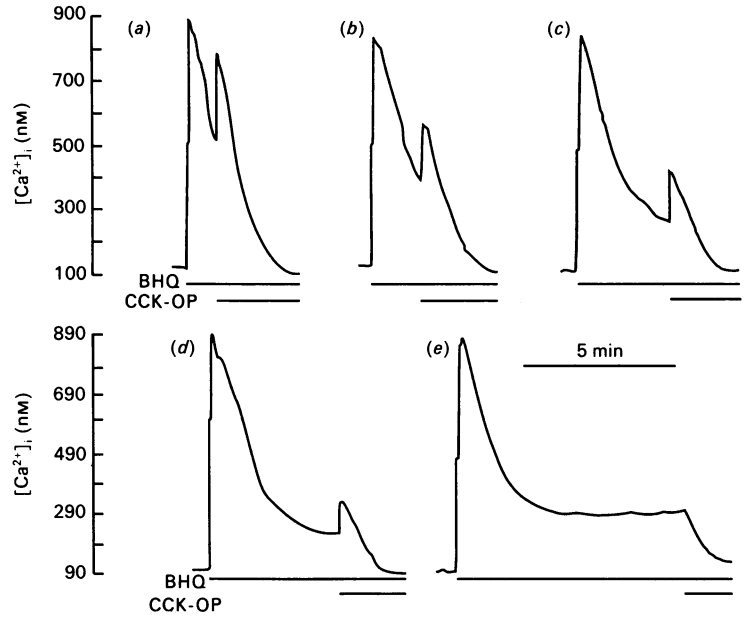

Fig. 5. Time course of inhibition of agonist-mediated $[Ca^{2+}]_i$ increase by BHQ

Separate cells were exposed to 25 μM-BHQ for 1 (*a*), 2 (*b*), 3 (*c*), 4.6 (*d*) or 7.5 (*e*) min before stimulation with 10 nM-CCK-OP. In all cases the signals were recorded from single cells. This represents an additional experiment in which the entire time course was performed and similar results were obtained.

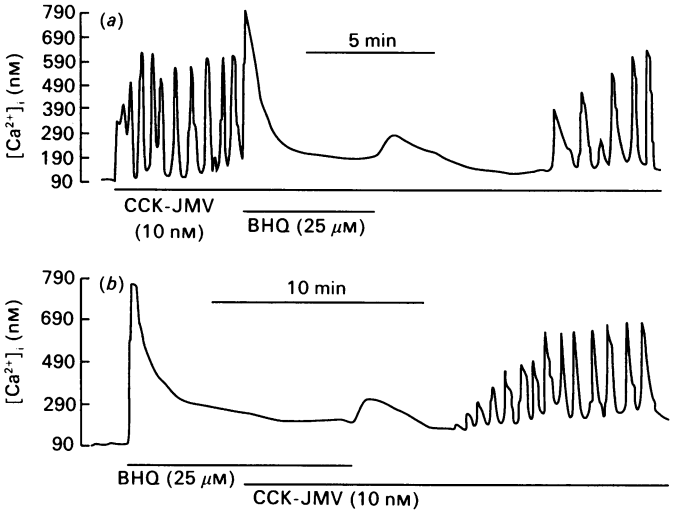

Fig. 6. Effect of BHQ on $[Ca^{2+}]_i$ oscillation

The cell in experiment (*a*) was continuously stimulated with 10 nM-CCK-JMV. Where indicated, 25 μM-BHQ was included in the perfusion medium. In experiment (*b*) the cell was exposed to 25 μM-BHQ and then to a solution containing BHQ and 10 nM-CCK-JMV, before removal of BHQ from the perfusion medium.

respectively. Incubation with BHQ for 7.5 min (Fig. 5*e*) was required to prevent the agonist-evoked $[Ca^{2+}]_i$ increase.

This relatively slow inhibition of the agonist-mediated response was reversible. To illustrate this, it was necessary to study a long-lasting effect of the agonist. Hence we monitored the effect of BHQ on $[Ca^{2+}]_i$ oscillation triggered by the CCK analogue CCK-JMV. We have previously shown that CCK-JMV stimulates long-lasting $[Ca^{2+}]_i$ oscillations in AR42J cells [27], similar to those observed in pancreatic acinar cells [28]. Fig. 6(*a*) shows that CCK-JMV induced $[Ca^{2+}]_i$ oscillation with an amplitude of 525 nM. Exposing such a cell to 25 μM-BHQ resulted in a rapid, transient, $[Ca^{2+}]_i$ and inhibition of further oscillation in $[Ca^{2+}]_i$. In addition, stimulation with CCK-JMV of a cell pretreated with BHQ had no further effect on $[Ca^{2+}]_i$ (Fig. 6*b*). Washing out the BHQ was followed by a small long-lasting transient increase in $[Ca^{2+}]_i$ and then a gradual recovery of $[Ca^{2+}]_i$ oscillation. The frequency of oscillation re-established almost immediately, but there was a time-dependent increase in amplitude. These experiments show that the slow action of BHQ on the agonist-mobilizable pool is not due to irreversible damage to the membrane of the intracellular stores.

The slow action of BHQ can be due to slow depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} pool. This aspect of the action of BHQ was measured by determining Ca^{2+} fluxes in permeabilized AR42J cells as shown in Fig. 7. We found that 25 μM-BHQ abolished ATP-dependent Ca^{2+} uptake in these saponin-treated cells. Addition of 10 μM-Ins(1,4,5)P_3 rapidly released about 60% (62 ± 12, $n = 3$) of the accumulated Ca^{2+}, followed by re-uptake as Ins(1,4,5)P_3 was depleted. On the other hand, 25 μM-BHQ caused slow Ca^{2+} efflux (with $t_{\frac{1}{2}}$ of about 150 s), and 5 min incubation with BHQ was required to deplete the Ins(1,4,5)P_3-mobilized Ca^{2+} pool. Fig. 7 also shows that BHQ did not inhibit the Ins(1,4,5)P_3-mediated Ca^{2+} release. When the two compounds were added together, Ins(1,4,5)P_3 still released Ca^{2+}, but there was no re-uptake as expected from the inhibition of the Ca^{2+} pump. The time course of depletion of the Ins(1,4,5)P_3-mobilizable Ca^{2+} pool by BHQ coincides with the inhibition of agonist-evoked $[Ca^{2+}]_i$ increase by BHQ (Fig. 5).

Effects of manoalide

Mobilization of intracellular Ca^{2+} by BHQ was attributed to its ability to inhibit the internal Ca^{2+} pump [12,26]. However, the results in Fig. 7 suggest that such inhibition leads to a slow rate of Ca^{2+} release, whereas in intact cells the BHQ-dependent $[Ca^{2+}]_i$ increase is completed within 4–5 s. Therefore it was decided to test the effect of the sesterterpenoid manoalide on the ability of agonists and BHQ to mobilize Ca^{2+}. We have previously

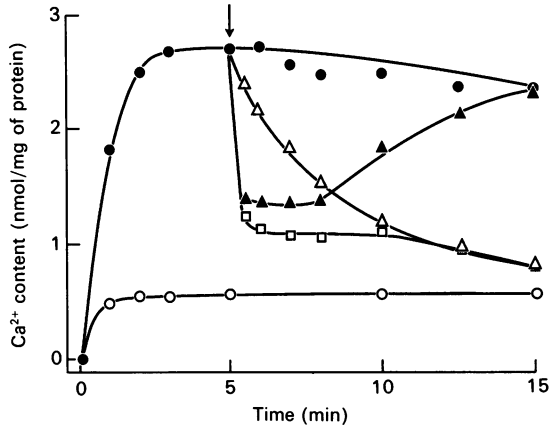

Fig. 7. Effect of BHQ and Ins(1,4,5)P_3 on Ca^{2+} uptake and release

AR42J cells were washed with uptake medium and permeabilized with saponin as detailed in the Methods section. The cells were used to measure Ca^{2+} uptake in the presence of ATP and in the absence (●) or presence (○) of 25 μM-BHQ. After 5 min incubation at 37 °C (arrow), samples of cells incubated in the absence of BHQ were transferred to tubes containing BHQ (△), Ins(1,4,5)P_3 (▲) or BHQ and Ins(1,4,5)P_3 (□) to give final concentrations of 25 μM-BHQ and 10 μM-Ins(1,4,5)P_3. At the indicated times, samples were removed and $^{45}Ca^{2+}$ content of the cells was determined. This experiment represents one of three with similar results.

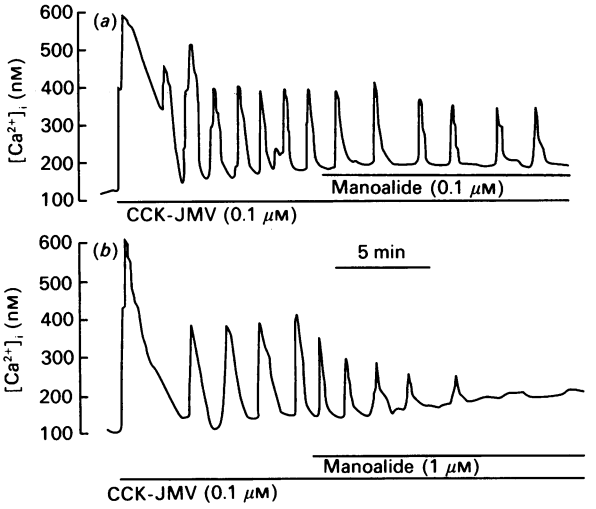

Fig. 8. Time- and concentration-dependence of manoalide effect on $[Ca^{2+}]_i$ oscillation

For these experiments the cells were perfused with BSA-free solution A to prevent binding of manoalide to BSA. In both experiments the cells were continuously stimulated with 0.1 μM-CCK-JMV to initiate $[Ca^{2+}]_i$ oscillation. When the oscillation became uniform, the cell in (*a*) was exposed to 0.1 μM-manoalide and the cell in (*b*) was exposed to 1 μM-manoalide. Note the effect of manoalide on frequency and amplitude of the oscillation.

shown that manoalide is a potent inhibitor of Ca^{2+} channels, including Ca^{2+} release from intracellular stores [29].

Fig. 8 shows that manoalide is able, dose-dependently, to inhibit $[Ca^{2+}]_i$ oscillation induced by CCK-JMV. At 0.1 μM-manoalide in the perfusion medium, there is a marked decrease in frequency and a small decrease in amplitude of the oscillation. Increasing manoalide concentration to 1 μM resulted in faster inhibition of $[Ca^{2+}]_i$ oscillation. In this case, manoalide decreased, in parallel, the frequency and amplitude of the oscillation.

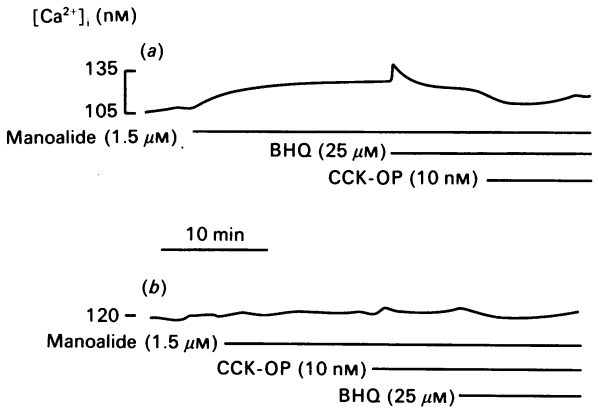

Fig. 9. Effect of manoalide on BHQ- and CCK-OP-evoked $[Ca^{2+}]_i$ increase

The cells were perfused with BSA-free solution A and treated with 1.5 μM-manoalide before exposure to 25 μM-BHQ and then 25 μM-BHQ and 10 nM-CCK-OP (*a*) or to CCK-OP and then CCK-OP and BHQ (*b*).

Complete inhibition of $[Ca^{2+}]_i$ changes occurred after 8 min incubation with manoalide. Pretreatment of the cells with 1 μM-manoalide for 10 min before stimulation completely prevented the $[Ca^{2+}]_i$ increase due to CCK-JMV (results not shown). The effect of manoalide probably reflects inhibition of agonist-stimulated Ca^{2+} release from intracellular stores and Ca^{2+} entry, since both pathways are required for $[Ca^{2+}]_i$ oscillation in this cell type [27] and both can be blocked by manoalide [29].

In the next set of experiments we tested the ability of manoalide to inhibit the BHQ-evoked $[Ca^{2+}]_i$ increase. Fig. 9(*a*) shows that preincubation of an AR42J cell with 1.5 μM-manoalide for 15 min had a very small effect on $[Ca^{2+}]_i$. This incubation almost completely inhibited the BHQ-mediated $[Ca^{2+}]_i$ increase. Similar incubation with manoalide also inhibited the effect of CCK-OP on $[Ca^{2+}]_i$ (Fig. 9*b*). As expected, in manoalide-treated cells CCK-OP added after BHQ (Fig. 9*a*) and BHQ added after CCK-OP had no effect on $[Ca^{2+}]_i$. Similar results were obtained when the experiments were repeated in the absence of Ca^{2+}_{out} (not shown).

In order to attribute the effect of manoalide to inhibition of Ca^{2+}-releasing pathways rather than the depletion of intracellular Ca^{2+} stores, it was necessary to show that the Ca^{2+} stores remained intact after pretreatment with manoalide. This was achieved by measuring the effect of manoalide on $[Ca^{2+}]_i$ increase induced by the ionophore ionomycin in the absence of Ca^{2+}_{out}. For these experiments we used cells in suspension, so that Ca^{2+} release from large number of cells could be tested. In addition, since the effect of the ionophore in Ca^{2+}-free medium is transient, measurements in cell suspension allow better estimation of the initial $[Ca^{2+}]_i$ change. Fig. 10(*a*) shows that after incubation of AR42J cells for 7.5 min in Ca^{2+}-free medium, 5 μM-ionomycin increased $[Ca^{2+}]_i$ from 140 to 1155 nM. Pretreatment of the cells with 1.5 μM-manoalide decreased the effect of ionomycin by only 15% (Fig. 10*b*), with an average of $14 \pm 3\%$ ($n = 4$). Similar pretreatment with manoalide abolished the effect of CCK-OP and BHQ on $[Ca^{2+}]_i$ (Fig. 10*c*), as was found in single-cell recording, with virtually no blunting of the ionomycin response. In additional control experiments, we tested directly whether manoalide interferes with either Ca^{2+} uptake into internal stores or the inhibition of internal Ca^{2+} pumps by BHQ. Fig. 11 shows that treatment of intact cells with 1.5 μM-manoalide and including manoalide in the permeabilization and uptake media had no inhibitory effect on Ca^{2+} uptake, in agreement with the experiments in Fig. 10. In addition, manoalide did not prevent

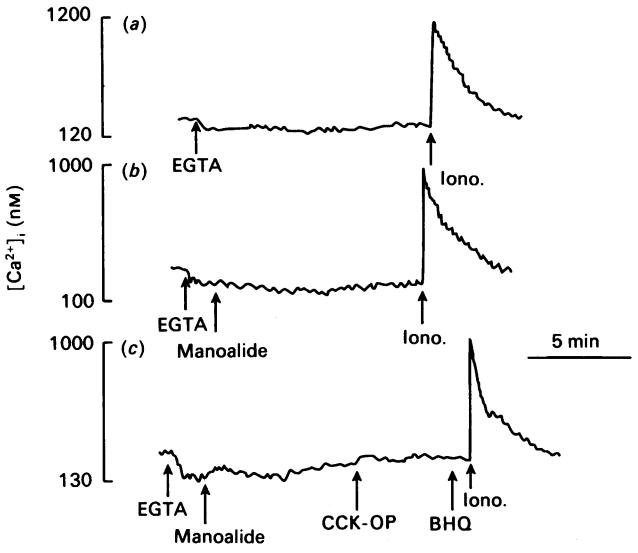

Fig. 10. Effect of manoalide on Ca^{2+} content of internal stores

AR42J cells were released from tissue-culture plates by trypsin/EDTA treatment, washed with solution A and loaded with Fura 2. A sample of cells (50 μl) was added to 2 ml of BSA-free solution A, to which no $CaCl_2$ was added. In (*a*) 0.1 mM-EGTA was added to the cuvette, and after 7.5 min 5 μM-ionomycin (Iono.) was added. In (*b*) 1.5 μM-manoalide was added 1 min after addition of EGTA, and where indicated 5 μM-ionomycin was added. In (*c*), after the incubation with EGTA and 1.5 μM-manoalide, the cells were exposed to 10 nM-CCK-OP and then to 25 μM-BHQ before addition of ionomycin.

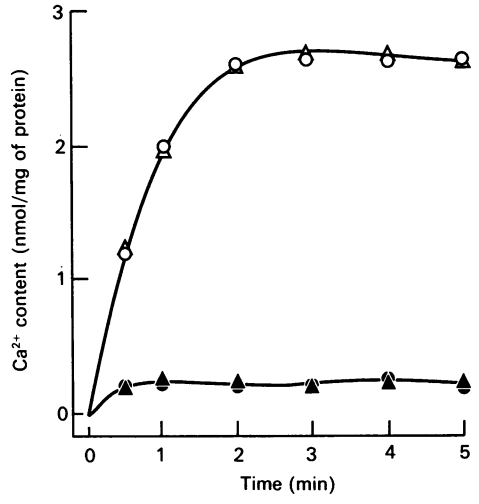

Fig. 11. Effect of manoalide on Ca^{2+}-pumping activity

AR42J cells suspended in BSA-free solution A were incubated with 1.5 μM-manoalide for 5 min at 37 °C. The cells were then washed with uptake medium, permeabilized with saponin and used for measurement of Ca^{2+} uptake as described in the Methods section and the legend to Fig. 7. All solutions used to test the effect of manoalide contained 1.5 μM of the drug. Control cells were treated with an equivalent volume of the solvent, dimethyl sulphoxide. The figure shows the effect of BHQ (●, ▲) on control (○, ●) and manoalide-treated (△, ▲) cells. The experiment shown represents one of three with similar results.

the inhibition of Ca^{2+} uptake by BHQ. The experiments in Figs. 10 and 11 further support our previous findings [29] showing that the effects of manoalide on $[Ca^{2+}]_i$ are due to inhibition of passive Ca^{2+} pathways.

Table 1. Mass measurement of Ins(1,4,5)P_3 in AR42J cells

AR42J cells grown on six-well plates were washed twice with solution A or BSA-free solution A (experiments with manoalide) and then treated with 10 nM-CCK-OP, 25 μM-BHQ or 1.5 μM-manoalide for the indicated times. Cells were also treated with 25 μM-BHQ or 1.5 μM-manoalide for 5 min before stimulation with 10 nM-CCK-OP. At the end of each incubation period, the reaction was terminated by aspiration of reaction medium and addition of 0.5 ml of 10% TCA. Ins(1,4,5)P_3 content in TCA extracts was determined as described in the Methods section.

		Ins(1,4,5)P_3 (pmol/well)		
Conditions	Time (s)...	5	10	300
Control		0.95±0.18	–	–
CCK-OP		10.21±0.20	12.15±0.21	–
BHQ		0.92±0.14	0.55±0.19	0.72±0.25
Manoalide		–	–	0.91±0.17
BHQ 5 min, CCK-OP		9.21±0.21	11.69±0.23	–
Manoalide 5 min, CCK-OP		10.66±0.24	12.33±0.20	–

Previous studies showed that BHQ [12] and manoalide [29] at the concentrations used had no effect on total production of InsP_3. To exclude the possibility that the drugs specifically inhibit production of the 1,4,5 isomer of InsP_3, we performed mass measurements of Ins(1,4,5)P_3 in resting and stimulated cells. Table 1 shows that in AR42J cells 5–10 s of stimulation with CCK-OP increased Ins(1,4,5)P_3 by approx. 12-fold. Incubation with 25 μM-BHQ for 5–300 s or with 1.5 μM-manoalide for 300 s had no measurable effect on Ins(1,4,5)P_3 levels. Similar incubation had no effect on the ability of CCK-OP to increase Ins(1,4,5)P_3 levels.

DISCUSSION

In the present study we used recording of $[Ca^{2+}]_i$ in single AR42J cells and pancreatic acini to determine the mode of action of BHQ on $[Ca^{2+}]_i$ in these cells. The single-cell recording system offers several advantages compared with cell suspensions. The rate of $[Ca^{2+}]_i$ increase due to BHQ can be determined more accurately. The shape of the $[Ca^{2+}]_i$ signal, whether transient or sustained, is better resolved. This is particularly significant with $[Ca^{2+}]_i$ measurements, where $[Ca^{2+}]_i$ changes stimulated by agonists appear monophasic when recorded in cell suspension but oscillating when recorded from single cells [4,6]. Measurements of the relationship between the agonist- and BHQ-releasable pools can be influenced by the presence of cells that do not respond to agonist but do respond to BHQ. For single cells, it is possible to circumvent this difficulty by selecting only agonist-responsive cells. Thus the use of single-cell recording methods as applied to the action of BHQ in AR42J cells has allowed a more precise definition of the mechanism of action of this useful compound and has provided evidence for separate pools of intracellular Ca^{2+} in this cell type.

In AR42J cells and pancreatic acini, BHQ increases $[Ca^{2+}]_i$ by Ca^{2+} release from intracellular stores and by increasing the plasma-membrane permeability to Ca^{2+}. Hence, BHQ caused a rapid $[Ca^{2+}]_i$ increase in the presence and absence of Ca^{2+}_{out}, indicating the mobilization of intracellular Ca^{2+}. Exposure of cells treated with BHQ in Ca^{2+}-free medium to 1 mM-Ca^{2+}_{out} was followed by a $[Ca^{2+}]_i$ increase blockable by La^{3+}. In addition, BHQ increased the rate of Mn^{2+} entry into these cells. Several studies have shown that Mn^{2+} can be used as a Ca^{2+} congener in passive Ca^{2+} pathways [30–32]. Thus Mn^{2+} enters the cells

through receptor-operated Ca^{2+} entry pathways and quenches fura 2 fluorescence when recorded at an excitation wavelength of 360 or 380 nm. The signal recorded with excitation at 360 nm is independent of Ca^{2+} [20], so that Mn^{2+} entry can be monitored without interference from the change in $[Ca^{2+}]_i$ [30,31]. The experiments with La^{3+} and Mn^{2+} provide direct evidence for augmentation of Ca^{2+} entry by BHQ. Our findings are quite different from those reported in hepatocytes. Measurements of $[Ca^{2+}]_i$ in suspension of hepatocytes showed that in the presence of $Ca^{2+}{}_{out}$ BHQ induced sustained, rather than transient, increase in $[Ca^{2+}]_i$. However, BHQ had no effect on Mn^{2+} entry [12,33]. These observations were interpreted to suggest that BHQ mobilizes Ca^{2+} from intracellular stores without a change in plasma-membrane permeability to Ca^{2+} [12,26,33]. The transient (Figs. 1 and 3) as compared with the sustained [12,26] nature of the response in the two studies might be explained by the recording system used. It is more difficult to account for the results with Mn^{2+}. We have no immediate explanation for these differences although it is possible that the Ca^{2+}-entry pathways activated by BHQ in hepatocytes do not transport Mn^{2+} in a manner similar to that in AR42J cells and pancreatic acini.

The effects of BHQ on $[Ca^{2+}]_i$ in AR42J cells resemble the effects described for thapsigargin in several cell types. Thapsigargin mobilized Ca^{2+} from intracellular stores [11,15,16], caused sustained increase in $[Ca^{2+}]_i$ [15,17] and increased Mn^{2+} influx [34]. Mobilization of intracellular Ca^{2+} by BHQ and thapsigargin did not involve a change in Ins(1,4,5)P_3 ([12,17], and the present work). However, the separation of intracellular Ca^{2+} into two distinct pools has not been described previously by using this class of reagents. Our results cannot be interpreted as direct activation of Ca^{2+} entry by BHQ. It is possible that Ca^{2+} release from intracellular pools was sufficient to trigger Ca^{2+} entry. It was shown in several cell types that mobilization of intracellular Ca^{2+} through receptor-mediated [32,35–37] or receptor-independent [17,34] means was sufficient to activate Ca^{2+} entry, which persisted also after removal of the agonist [32,35–37]. If this mechanism accounts for the activation of Ca^{2+} entry by BHQ, then our results suggest that Ca^{2+} release from either the agonist-sensitive or the agonist-insensitive pools can activate Ca^{2+} entry in AR42J cells and pancreatic acini.

Our results suggest that BHQ treatment leads to Ca^{2+} release from two separate pools, an agonist-sensitive and an agonist-insensitive pool. Ca^{2+} release from the agonist-insensitive pool is rapid and responsible for the initial BHQ-mediated $[Ca^{2+}]_i$ increase, whereas depletion of the agonist-sensitive pool by BHQ is slow. This is concluded from the following: (*a*) exposure of cells to BHQ and agonist increased $[Ca^{2+}]_i$ to levels higher than those observed with either BHQ or the agonist (Fig. 4*b*); (*b*) BHQ can still increase $[Ca^{2+}]_i$ in cells whose agonist-mobilizable pool has been depleted (Fig. 4*a*); (*c*) BHQ induced an initial rapid $[Ca^{2+}]_i$ increase through a manoalide-sensitive Ca^{2+} pathway. Even when this release is complete, agonists are still able to mobilize Ca^{2+} (Fig. 5). Complete inhibition of agonist-evoked $[Ca^{2+}]_i$ increase by BHQ required 5–7 min incubation of 37 °C. Since BHQ is a potent inhibitor of intracellular Ca^{2+} pumps ([14], and Figs. 7 and 11 for AR42J cells), it is likely that the slow time-dependent inhibition of the agonist response is related to a slow leak of Ca^{2+} from the agonist-mobilizable pool. This interpretation of the results is supported by the studies in permeabilized cells, in which inhibition of the pump by BHQ resulted in slow leak of Ca^{2+} from the Ins(1,4,5)P_3-mobilizable pool. Our results therefore suggest that most of the Ca^{2+} rapidly mobilized by BHQ is from a pool separate from the agonist-mobilizable pool while mobilization of Ca^{2+} from the agonist-sensitive pool by BHQ is relatively slow, and correlates with inhibition of the Ca^{2+} pump in this pool.

An interesting finding in the present studies is that the initial and rapid mobilization of Ca^{2+} by BHQ can be blocked by manoalide, although manoalide does not interfere with inhibition of internal Ca^{2+} pumps by BHQ. It was previously shown that BHQ is a potent and selective inhibitor of internal membrane Ca^{2+} pumps [12,14] and that it had no effect on passive Ca^{2+} fluxes in these membranes [14]. Hence it was suggested that the $[Ca^{2+}]_i$ increase by BHQ in intact cells is due to inhibition of the internal-stores Ca^{2+} pump and non-specific leak of Ca^{2+} from the stores. We suspected that Ca^{2+} release due to BHQ probably involves Ca^{2+} efflux through a specific channel when it was found that the maximal $[Ca^{2+}]_i$ increase induced by BHQ is completed within 4–5 s, whereas Ca^{2+} release from internal stores of permeabilized cells required 5 min (Fig. 7). However, the most compelling evidence for the involvement of Ca^{2+} channels in the initial BHQ-mediated Ca^{2+} release was obtained with the use of manoalide. Manoalide is a potent inhibitor of various types of Ca^{2+} channels, including agonist-mediated Ca^{2+} release from internal stores and Ca^{2+} entry [29]. We also show here that manoalide at the concentrations used does not inhibit the internal-stores Ca^{2+} pumps. Yet manoalide inhibited the effect of BHQ and the agonist on $[Ca^{2+}]_i$. Of course, if BHQ was to increase $[Ca^{2+}]_i$ owing to pump inhibition followed by a non-specific Ca^{2+} leak, then manoalide was expected to have no effect on the BHQ response and to inhibit only the response to the agonists.

Although the data presented argue for involvement of a Ca^{2+} channel in the BHQ-evoked Ca^{2+} release, it does not indicate direct activation of the channels by BHQ. It is possible that inhibition of the Ca^{2+} pumps in the agonist-insensitive pool by BHQ unmasked the activity of a Ca^{2+} channel present in the membrane of this pool. In this case, we must assume a very rapid pump-leak turnover of Ca^{2+} across the membrane of this pool. Alternatively, inhibition of the Ca^{2+} pumps by BHQ can result in small Ca^{2+} efflux from this pool, and the released Ca^{2+} can then activate the Ca^{2+} channel, possibly by a Ca^{2+}-dependent Ca^{2+}-releasing mechanism. Such a mechanism, or any other indirect mechanism for activation of the Ca^{2+} channels following pump inhibition by BHQ will remove the necessity for rapid pump-leak turnover, which is unfavourable energetically. The slow Ca^{2+} release from the agonist-sensitive pool by BHQ probably reflects a low concentration of Ins(1,4,5)P_3 at the site of Ca^{2+} release. In this case, inhibition of the Ca^{2+} pumps in this pool results in slow Ca^{2+} release and time-dependent inhibition of the response to agonist.

The nature of the Ca^{2+} pool rapidly mobilized by BHQ is not known at present. However, it was shown that multiple Ca^{2+} pools exist in cells, and Ins(1,4,5)P_3 mobilizes Ca^{2+} only from some of these pools [4–6]. Therefore it is possible that the Ca^{2+} rapidly mobilized by BHQ is stored in this fraction of the endoplasmic reticulum. We could not substantiate such a possibility by measuring the effect of BHQ and Ins(1,4,5)P_3 on Ca^{2+} release in permeabilized cells. Although Ins(1,4,5)P_3 induced rapid Ca^{2+} release and its effect was not blocked by BHQ, we could not demonstrate similar rapid Ca^{2+} release by BHQ. A possible explanation for this is a loss of the mechanism of Ca^{2+} release triggered by BHQ in permeabilized cells. In this context, it is noteworthy that there is some evidence for Ca^{2+}-mediated Ca^{2+} release in intact pancreatic acini [38,39], although similar activity could not be demonstrated in permeabilized cells. It is therefore possible that the BHQ-releasable pool may be responsible in pancreatic acinar cells for Ca^{2+}-induced Ca^{2+} release in a mechanism described above.

BHQ completely and rapidly prevented the $[Ca^{2+}]_i$ oscillation. This suggests that continuous operation of the Ca^{2+} pumps and Ca^{2+} fluxes across the membranes of the intracellular pools are

required for re-loading of the pools during the oscillation. In all experiments tested ($n = 7$), removal of BHQ was followed by slow and small transient $[Ca^{2+}]_i$ increase before the oscillation resumed. This transient may reflect an increased Ca^{2+} influx or decreased efflux across the plasma membrane before maximal recovery of Ca^{2+} uptake by intracellular Ca^{2+} pumps. Once inhibition of the pumps is completely relieved, the pool can re-load with Ca^{2+} and the oscillation will start only when the Ca^{2+} content of the pool reaches a critical level. Such regulation may be related to a recent suggestion that Ca^{2+} content in the pool regulates the affinity for Ins(1,4,5)P_3 and the Ca^{2+} release [40].

In summary, BHQ has multiple actions on the $[Ca^{2+}]_i$ regulatory mechanisms of AR42J cells and pancreatic acini. The compound, as demonstrated in hepatocytes, is able to inhibit the Ca^{2+} pump re-loading the Ins(1,4,5)P_3-releasable pool of Ca^{2+}, and thus to blunt gradually the response of these cells to Ca^{2+}-mobilizing agonists. In this way, the compound also inhibits $[Ca^{2+}]_i$ oscillations induced in this cell type by CCK-JMV. A rapid $[Ca^{2+}]_i$ transient induced by BHQ is best explained by the presence of an additional Ca^{2+} pool that can be released by BHQ through inhibition of the pumps in this second pool and unmasking the activity of a manoalide-sensitive passive Ca^{2+} pathway. The release through this pathway is independent of changes in Ins(1,4,5)P_3 concentrations. In addition, BHQ is able to activate a La^{3+}-inhibitable Ca^{2+}-entry pathway that also admits Mn^{2+}. Activation of this pathway may relate to Ca^{2+} release from the internal pools.

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